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# Butyrate produced by commensal bacteria down-regulates indolamine 2, 3-dioxygenase 1 (IDO-1) expression via a dual mechanism in human intestinal epithelial cells.

Camille Martin-Gallausiaux<sup>1, 2</sup>, Pierre Larraufie<sup>1, 3</sup>, Anne Jarry<sup>4, 5</sup>, Fabienne Béguet-Crespel<sup>1</sup>, Ludovica Marinelli<sup>1, 2</sup>, Florence Ledue<sup>1</sup>, Frank Reimann<sup>3</sup>, Hervé M. BLOTTIERE<sup>1, 6</sup>, Nicolas Lapaque<sup>1\*</sup>

<sup>1</sup>Institut Micalis, France, <sup>2</sup>Sorbonne Universités, France, <sup>3</sup>MRC Metabolic Diseases Unit, Metabolic Research Laboratories, University of Cambridge, United Kingdom, <sup>4</sup>INSERM U1232 Centre de Recherche en Cancérologie et Immunologie Nantes Angers (CRCINA), France, <sup>5</sup>University of Nantes, France, <sup>6</sup>MetaGenoPolis (MGP), Université Paris-Saclay, France



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#### Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

#### Author contribution statement

Conceived and designed the experiments: CMG, NL; performed most of the experiments: CMG; performed some experiments: PL, AJ, FBC, LM, FL, NL; analysed the data: CMG, AJ, NL; contributed materials: FR; wrote the paper: CMG, NL; edited and revised the manuscript: AJ, PL, FR, HMB.

#### Keywords

Gut Microbiota, ido-1, Intestinal epithelial cell (IEC), Butyrate, commensal bacteria, host-microbiota crosstalk, Immune gene regulation

#### Abstract

#### Word count: 274

Commensal bacteria are crucial for the development and maintenance of a healthy immune system therefore contributing to the global wellbeing of their host. A wide variety of metabolites produced by commensal bacteria are influencing host health but the characterisation of the multiple molecular mechanisms involved in host-microbiota interactions is still only partially unravelled. The intestinal epithelial cells (IEC) take a central part in the host-microbiota dialogue by inducing the first microbial-derived immune signals. Amongst the numerous effector molecules modulating the immune responses produced by IECs, indoleamine 2,3-dioxygenase-1 (IDO-1) is essential for gut homeostasis. IDO-1 expression is dependent on the microbiota and despites its central role, how the commensal bacteria impacts its expression is still unclear. Therefore, we investigated the impact of individual cultivable commensal bacteria on IDO-1 transcriptional expression and found that the short chain fatty acid (SCFA) butyrate was the main metabolite controlling IDO-1 expression in human primary IECs and IEC cell-lines. This butyrate-driven effect was independent of the G-protein coupled receptors GPR41, GPR43 and GPR109a and of the transcription factors SP1, AP1 and PPARγ for which binding sites were reported in the IDO-1 promoter. We demonstrated for the first time that butyrate represses IDO-1 expression by two distinct mechanisms. Firstly, butyrate decreases STAT1 expression leading to the inhibition of the IFNγ-dependent and phosphoSTAT1-driven transcription of IDO-1. In addition, we described a second mechanism by which butyrate impairs IDO-1 transcription in a STAT1-independent manner that could be attributed to its histone deacetylase (HDAC) inhibitor property.

In conclusion, our results showed that IDO-1 expression is down-regulated by butyrate via a dual mechanism: the reduction of STAT1 level and the HDAC inhibitor property of SCFAs.

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Primary colonocytes cultures: Human tissues were obtained from the Human Research Tissue Bank at the Addenbrooke's hospital, Cambridge under the license 09/H0308/24.

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7	Lapaque <sup>1*</sup> .
8	
9	
10	<sup>1</sup> Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France;
11	<sup>2</sup> Sorbonne Universités, UPMC Univ Paris 06, IFD, Paris, France.
12	<sup>3</sup> University of Cambridge, Metabolic Research Laboratories and MRC Metabolic Diseases
13	Unit, WT-MRC Institute of Metabolic Science, Addenbrooke's Hospital, Cambridge, UK;
14	<sup>4</sup> INSERM UMR 1232, Université de Nantes, Nantes, France;
15	<sup>5</sup> US 1367 MetaGenoPolis, INRA, Université Paris-Saclay, Jouy en Josas, France.
16	
17	* Corresponding author: Dr Nicolas Lapaque
18	INRA-MICALIS UMR1319, Bat 442, Domaine de Vilvert 78350, Jouy-en-Josas, France.
19	e-mail: nicolas.lapaque@inra.fr
20	
21	Keywords: Gut microbiota, IDO-1, intestinal epithelial cells, butyrate, Immune gene
22	regulation.
23	

#### 24 Abstract:

25 Commensal bacteria are crucial for the development and maintenance of a healthy immune system therefore contributing to the global wellbeing of their host. A wide variety of 26 27 metabolites produced by commensal bacteria are influencing host health but the characterisation of the multiple molecular mechanisms involved in host-microbiota 28 29 interactions is still only partially unravelled. The intestinal epithelial cells (IEC) take a central 30 part in the host-microbiota dialogue by inducing the first microbial-derived immune signals. 31 Amongst the numerous effector molecules modulating the immune responses produced by 32 IECs, indoleamine 2,3-dioxygenase-1 (IDO-1) is essential for gut homeostasis. IDO-1 33 expression is dependent on the microbiota and despites its central role, how the commensal 34 bacteria impacts its expression is still unclear. Therefore, we investigated the impact of 35 individual cultivable commensal bacteria on IDO-1 transcriptional expression and found that 36 the short chain fatty acid (SCFA) butyrate was the main metabolite controlling IDO-1 expression in human primary IECs and IEC cell-lines. This butyrate-driven effect was 37 38 independent of the G-protein coupled receptors GPR41, GPR43 and GPR109a and of the transcription factors SP1, AP1 and PPARy for which binding sites were reported in the IDO-1 39 40 promoter. We demonstrated for the first time that butyrate represses *IDO-1* expression by two 41 distinct mechanisms. Firstly, butyrate decreases STAT1 expression leading to the inhibition 42 of the IFNy-dependent and phosphoSTAT1-driven transcription of IDO-1. In addition, we 43 described a second mechanism by which butyrate impairs IDO-1 transcription in a STAT1-44 independent manner that could be attributed to its histone deacetylase (HDAC) inhibitor 45 property.

In conclusion, our results showed that *IDO-1* expression is down-regulated by butyrate *via* a
dual mechanism: the reduction of STAT1 level and the HDAC inhibitor property of SCFAs.

#### 48 Introduction

49 The gut microbiome is a microbial ecosystem that exerts diverse functions often associated with beneficial physiological effects for its host. Among these essential functions, 50 51 the intestinal microbiome provides an extended repertoire of molecules that influences the 52 host health notably via the development and the maturation of its immune system (Sekirov et 53 al., 2010; Postler and Ghosh, 2017). The molecular bases of the host-microbiota interactions 54 are only just beginning to be unravelled and are mediated by a wide variety of metabolites 55 produced by commensal bacteria (Blacher et al., 2017; Postler and Ghosh, 2017). Many bacteria-derived metabolites originate from dietary sources. Among them, an important role 56 57 has been attributed to the metabolites derived from the bacterial fermentation of dietary fibres, 58 namely the short chain fatty acids (SCFAs) linking host nutrition to immune development and functions (Blacher et al., 2017; Postler and Ghosh, 2017). Human cells respond to SCFAs 59 through a signalling activation cascade involving specific G-protein coupled receptors 60 (GPR41, GPR43 and GPR109a) and through an epigenetic regulation of gene expression by 61 the inhibition of lysine or histone deacetylases (HDACs) (Hinnebusch et al., 2002; 62 63 Schilderink et al., 2013; Fellows et al., 2018).

64 Numerous studies suggest that the close intimacy between the mucosal microbial populations and the host intestinal cells is central for the fine regulation of the host 65 66 physiology. Indeed, intestinal epithelial cells (IEC) provide a crucial physical barrier against harmful pathogens and are also key players in the initiation and maintenance of mucosal 67 68 immune responses (Kagnoff, 2014). Accordingly, indigenous members of the microbiota have 69 dramatic and specific impacts on the host immune system through their intimate interactions with the host epithelium (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Atarashi et al., 70 71 2011; Schilderink et al., 2013; Atarashi et al., 2015).

72 Indoleamine 2,3-dioxygenase-1 (IDO-1) is an enzyme that catalyses the oxidation of 73 the indole moiety of the essential amino acid tryptophan leading to production of N-formyl-74 kynurenine and its derivatives. In the last decades, a growing number of studies showed the 75 importance of IDO-1 in various pathologies, including, autoimmune diseases, allergy and cancer (Platten et al., 2005; Munn and Mellor, 2007). Despites the fact that IDO-1 expression 76 77 was largely thought to be protective, several recent studies suggest a detrimental role of IDO-78 1 expression in obesity, atherosclerosis, vascular inflammation and aneurysm (Metghalchi et 79 al., 2015; Laurans et al., 2018; Metghalchi et al., 2018). These results suggest that IDO-1 80 plays a far more complex role in health and fine-tuning of its expression and activity might 81 occur in healthy individuals. Mechanisms inducing IDO-1 expression during inflammation 82 have already been described and include IFNy and type-I IFN. However, natural factors 83 inhibiting IDO-1 expression have not been reported yet.

84 The gut, along with the skin, is a major site of IDO-1 activity at steady state. IDO-1 85 expression in human healthy IECs is poorly described but has been reported in several studies 86 to be increased in IBD (Barcelo-Batllori et al., 2002; Ferdinande et al., 2008; Cherayil, 2009; 87 Zhou et al., 2012). In the murine gut, its expression is dependent on the microbiota (Rhee et 88 al., 2005; Atarashi et al., 2011). These observations prompted us to investigate the impact of 89 individual cultivable commensal bacteria on *IDO-1* transcriptional expression. In the current 90 study, we screened over 401 bacterial supernatants on an IDO-1 reporter system and found 91 that butyrate was the main inhibitor of IDO-1 expression in human primary IECs and cell-92 lines. The IDO-1 down-regulation was independent of GPR41, GPR43 and GPR109a, three 93 known G-protein coupled receptors for SCFAs and of SP1, AP-1 and PPARy, three 94 transcription factors targeted by butyrate and for which binding sites were reported in the 95 IDO-1 promoter. Our results showed that butyrate regulated IDO-1 expression via a dual 96 mechanism. First, butyrate decreased STAT1 expression leading to the inhibition of the IFNy-

97 dependent phosphorylation of STAT1 and consequently the STAT1-driven transcriptional
98 activity of *IDO-1*. In addition, we described a second mechanism by which butyrate impaired
99 *IDO-1* transcription in a STAT1 independent manner that could be attributed to the HDAC
100 inhibitory property of SCFAs.



### 102 Materials and methods

#### 103 **IDO-1** expression in human normal colon at the protein and mRNA levels

104 Macroscopically and microscopically unaffected human normal colon was obtained from 10 105 patients undergoing surgery for colon cancer, at least at 10 cm downstream the tumour [7 106 men, 3 women; mean age 62 years; left (7) or right colon (3)]. The tissue fragments were 107 processed accordingly to the French guidelines for research on human tissues, including 108 patients' consent. IDO-1 immunostaining was performed using a monoclonal antibody (clone 109 4D2, Serotec) and a standard streptavidin-biotin- peroxidase technique after antigen retrieval 110 in citrate buffer pH6. Diaminobenzidine was used as a chromogen and nuclei were 111 counterstained with hematoxylin. IDO-1 mRNA levels were assessed on preparations of 112 isolated IECs after EDTA treatment and on whole mucosa microdissected from the normal colon as previously described (Jarry et al., 2008). Samples were prepared by beads-beating 113 114 mechanical lysis using Fastprep (MP Biomedicals) and centrifuged at 8,000g for 10 min at 115 4°C prior RNA extraction and RT-PCR analysis.

# 116 Cell Culture of human intestinal cell lines and primary colonocytes

117 The human epithelial cell lines HT-29 and Caco-2 were obtained from the American Type 118 Culture Collection (ATCC, Rockville, MD) and grown as described (Martin-Gallausiaux et 119 al., 2018). Four human primary colonic cell culture from three different donors were performed as described (Habib et al., 2013). Briefly, PBS-washed colonic tissues were 120 121 digested with 0.5mg/ml of collagenase type XI. The crypts were plated onto Matrigel coated 122 plates and cultured for 24h in DMEM 24mM glucose supplemented with 10% FCS, 2mM L-123 Glutamine, 50 U/mL penicillin, 50 U/mL streptomycin and Y-27632 (Tocris). The day after 124 plating, media was rinsed with fresh media and replaced with culture media with or without butyrate 2mM. Human tissues were obtained from the Human Research Tissue Bank at the
Addenbrooke's hospital, Cambridge under the license 09/H0308/24.

## 127 Luciferase Reporter and cell viability Assays

128 A 1.6-Kb section of the human *IDO-1* promoter was cloned using KpnI and NheI restrictions 129 sites (Primers used were Fw: AAAGGTACCGGGTAGGATAGATTTAGTGAG; Rv: 130 AAAAAGCTAGCCATTCTTGTAGTCTGCTCC) into the pGL4.14 (Promega) luciferase 131 plasmid and used to establish the stable HT-29 IDO-1 reporter cell-line after antibiotic selection (hygromycin, 600 µg/mL, InvivoGen) and validated with IFNy (100U/mL, 132 Peprotech) and IL1β (10ng/mL, Peprotech). For each experiment, HT-29-*IDO-1* reporter cells 133 were seeded at  $3x10^4$  cells per well in 96-well plates 24h prior to incubation with bacterial 134 135 supernatants or reagents. The cells were stimulated for 24h with 10 µL of bacterial supernatants in a total culture volume of 100 µL per well (i.e., 10% vol/vol) prior to the 136 137 luciferase assay. The luciferase activity was quantified as relative luminescence units using a 138 microplate reader (Tecan) and the Neolite Luminescence Reporter Assay (Perkin-Elmer) 139 according to the manufacturers' instructions. The IDO-1 activity was normalized to the 140 controls, i.e., the un-stimulated cells or cells in presence of non-inoculated bacteria culture 141 medium. Experiments were performed in triplicates for at least three biological independent 142 assays. Cell viability was monitored by MTS measurement using the CellTiter 96 Aqueous 143 One solution (Promega) according to the manufacturer's recommendations.

# 144 Culture of commensal bacteria, preparation of supernatants and SCFAs concentration 145 assessment.

146 135 human intestinal commensal bacterial strains which include 111 different species from147 the in-house INRA-Micalis collection or from DSMZ were grown. Bacterial cultures and

supernatants were performed as described (Martin-Gallausiaux et al., 2018). Screened species
and strains, corresponding growth media, optical densities (OD), short chain fatty acids
(SCFAs) concentrations are listed in Supplementary Table 1. Concentrations of SCFAs
produced by cultured bacteria were measured by HPLC and gas chromatography as described
(Bourriaud et al., 2005).

#### 153 **Reagents and cytokines**

154 All agonists, drugs and inhibitors were dissolved in glycerol, DMSO or water. Sodium salt of 155 SCFAs were from Sigma and used in a range of concentrations from 0.5 to 8mM. GPRs 156 agonists: GPR41: 4-CMTB (1µM Tocris) and Tiglic acid (1-10mM Sigma); GPR43: 157 AR420626 (1µM Cayman) and 1-MCPC (1mM Sigma); GPR109a: Niacine (1mM-10mM, 158 Sigma) and MK1903 (1µM Tocris). GPRs sub-unit inhibitors used were: Pertussis toxin (Ptx 159 0.2µg/ml) and U73122 (10 µM) from Sigma. HDAC inhibitors: Trichostatin A (TSA 1µM 160 Sigma), SAHA (5µM Sigma) and valproic acid (VPA 5mM Sigma). SP1 inhibitor 161 Mithramycin A (0.1µM Sigma). PPARy activators: Pioglitazone (5µM), Roziglitazone 162 (10µM) and PPARy inhibitor G9662 (100µM), from Cayman. NF-kB inhibitor BAY 11-7082 163 (40µM). AP-1 inhibitor SR-11302 (10µM Tocris). STAT3/Jak2 inhibitor Cucurbitacin I (1µM) from Tocris. IFNy (100U/ml) and TNFa (10ng/ml) were from Peprotech. Final 164 165 concentration of DMSO had no detectable effect on cells viability or responses.

166

#### 167 **Plasmids and transfection**

168 Human GPR43 and GPR109a were cloned after EcoRI and XhoI digestion in pCMV-eGFP-169 N1 vector. Oligonucleotides used for amplification of GPR43 were 170 aaaactcgagatgctgccggactggaa and aaaagaattcctactctgtagtgaagtccga. Oligonucleotides used 171 for amplification of GPR109a were aaaactcgagatgaatcggcaccatctgcaggat and

*aaaagaattcttaaggagaggttgggcccaga*. HT-29 cells were seeded at 3.10<sup>4</sup> density per well in 96well plates and transiently transfected with Lipofectamine 2000 (Thermofischer). 24h after
transfection, incubation with reagents was done for an additional 24h prior luciferase activity
measurement.

#### 176 siRNA assays

HT29 cells were seeded at 4.10<sup>5</sup> cells per well in a 6 wells plates on day 1 and siRNA were
transfected with DharmaFect I at final concentrations of 1 and 25nM on day 2 and 3,
following the manufacturer's instructions (Dharmacom). Incubation with drugs was done on
day 6 and *IDO-1* activity was assessed on day 7. siRNA SMARTpool ON-TARGETplus
STAT1 siRNA (L-003543-00-0005) and ON-TARGETplus Non-targeting Pool (D-00181010-05) were from Dharmacon.

## 183 **Real-Time PCR**

184 Real-Time PCR were performed as described (Martin-Gallausiaux et al., 2018). qPCRs were 185 carried out using a StepOnePlus Real-Time PCR System (ThermoFischer Scientific) with 186 Taqman gene expression assay probes : GAPDH Hs02758991 g1, IDO-1 Hs00984148 m1, GPR43 Hs00271142 s1, GPR41 Hs02519193 g1, GPR109a Hs02341584 s1, RBP1 187 Hs01011512 g1, Actinbeta Hs99999903 m1, STATI s01013996 m1, B2M Hs99999907 m1. 188 189 GAPDH, Actin, RBP1 and B2M were used for internal normalisation. Samples were tested in experimental duplicates and at least in biological triplicates. For primary cells treated with 190 191 butyrate and control, cDNAs were pre-amplified (10 cycles) using the TaqMan PreAmp 192 Matster Mix Kit following the manufacturer's recommendations.

### 193 Western blot analysis

HT-29 cells were seeded at densities of  $5 \times 10^5$  cells per well in 24-well-plates for 24h prior 194 stimulation. Cells were washed twice and lysed in buffer (1% NP40, 150mM NaCl, 50mM 195 196 Tris-HCL pH8, 5mM EDTA, 1 x Complete Protease Inhibitor Cocktail (Roche), 1X x Phos 197 STOP phosphatase Inhibitor Cocktail (Roche). Nucleus were eliminated by centrifugation for 198 10 minutes 4°C at 17500g. Protein extracts were run in SDS-PAGE gels and transferred onto 199 PVDF membranes. Membranes were blocked overnight in TBS 0.1% tween 4% skim milk or 200 BSA (Sigma). Primary antibodies were incubated overnight at 4°C (STAT1 1:1000 (D1K9Y), STAT1-phospho TYR 701 1:1000 (58D6), STAT3 1:1000 (124H6), Lamin A/C 1:2000 201 202 (4C11) all from Cell signalling; Actin 1:2000 (AC-40) from Sigma, GAPDH 1:2000 from Santa Cruz). Secondary (Goat anti-Rabbit IgG HRP (P0448) and Goat anti-mousse HRP 203 204 (P0447) from Dako) antibodies were successively added for 1h before detection with the 205 Clarity Western ECL Substrate using the Chemidoc MP System (Bio-Rad). Quantifications 206 were performed using the image Lab software (Bio-Rad). Proteins levels were internally 207 normalised with GAPDH or Actin before comparison with experimental controls.

#### 208 Cytoplasmic and nuclear proteins extraction

HT-29 cells were seeded at densities of  $5 \times 10^5$  cells/well in 24-well-plates for 24h prior stimulation with butyrate. Cytoplasmic and nuclear protein extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (ThermoScientific). Lamin A/C and GAPDH were used as nuclear and cytoplasmic protein loading controls respectively.

## 214 **Promoter analysis**

*In silico* analysis of the promoter sequence upstream of the transcription start of *IDO-1* was
performed using Genomatix MatInspector software (core similarity=1; matrix similarity
>0.8).

## 218 Statistical Analysis

Data were analysed using R and RStudio software. Function for PCA analysis: prcomp.
Correlation matrix was done with Hmisc package. Graphics were produced with ggplot2
package and Prism GraphPad software. Statistical analysis was done with Student two-sided
test or Wilcoxon rank test.

#### 224 **Results**

#### **IDO-1** is expressed in epithelial cells of the human normal colonic mucosa

226 IDO-1 expression is well documented in dendritic cells (DC) and macrophages(Matteoli et al., 227 2010). However its expression in intestinal epithelial cells (IECs) has been scarcely studied in 228 human. We assessed IDO-1 expression both by immunohistochemistry on paraffin sections of 229 normal human colonic mucosa (n=10) and at the mRNA level. In 8 cases, IDO-1 was 230 expressed by IECs with either strong homogeneous staining of more than 80% IECs all along 231 the colonic crypts (perinuclear and/or membrane staining in enterocytes and goblet cells; 232 Figure 1A, left panel) or heterogeneous staining of IECs (10-20% of IECs; Figure 1A, right 233 panel). In 2 samples, IDO-1 was barely detectable in IECs. IDO-1 was also expressed in the 234 lamina propria, in some mononuclear cells and endothelial cells (Figure 1A). IDO-1 expression was then confirmed by RT-PCR on RNA extracted from preparations of isolated 235 236 human IECs from normal colon. As shown in Figure 1B, isolated human IECs expressed an 237 *IDO-1* level comparable with the expression level from the entire colonic mucosa suggesting 238 that IECs were an important source of IDO-1 mRNAs in the colon.

#### 239 Metabolites derived from commensal bacteria modulate *IDO-1* expression

In the gut, *IDO-1* expression is dependent on the microbiota since colonisation of mice with commensal bacteria induced high levels of *IDO-1* in IECs (Rhee et al., 2005; Atarashi et al., 2011). In an attempt to decipher which commensal bacteria influence *IDO-1* expression, we performed a screening with an *IDO-1* reporter system expressed in the human epithelial cell line HT-29. As recently reported in animal studies and in functional metagenomic studies, bioactive compounds produced by commensal bacteria are likely to be small-secreted molecules, we thus tested the bacterial supernatants of 135 members of the human microbiota 247 that include 60% of species close to the human core microbiome on an *IDO-1* reporter system 248 (Supplementary Table 1) (Qin et al., 2010; Cohen et al., 2015; Blacher et al., 2017; Postler 249 and Ghosh, 2017). In this set-up, only few bacterial supernatants were activating IDO-1 expression in HT-29 cells, including some Lactobacillaceae (Supplementary Figure 1). 250 251 Interestingly, a global and dramatic down-regulation of IDO-1 was observed in HT-29 252 challenged with supernatants of Firmicutes and Fusobacteria, while Actinobacteria, Bacteroidetes, Proteobacteria and Verrucomicrobia barely modulated IDO-1 expression 253 254 (Figure 2A, Supplementary Figure 1).

#### 255 Butyrate down-regulates *IDO-1* expression in epithelial cells

256 Among the Firmicutes, the most active genera on IDO-1 expression were Clostridium, 257 Lachnoclostridium, Ruminoclostridium and Roseburia (Supplementary Figure 1). All these 258 genera in addition to the Fusobacterium genus share a common active role in the diet-derived 259 fibre degradation leading to the production of short-chain fatty acids (SCFAs) by anaerobic 260 fermentation (Vital et al., 2014). We thus hypothesized that the down-regulated pattern of 261 IDO-1 expression could be explained by the SCFA concentration in the bacterial 262 supernatants. We therefore quantified the concentrations of acetate, propionate, butyrate, isobutyrate, valerate and isovalerate by GC-MS or HPLC in some bacterial supernatants 263 264 (Supplementary Table 1). Principal component (PCA) and correlation analyses on SCFAs 265 concentrations and IDO-1 activity showed a negative correlation between butyrate 266 concentration and IDO-1 expression (Figure 2B and Supplementary Figure 2A). Specific 267 impact of butyrate on *IDO-1* was confirmed by a pairwise spearman correlation (Figure 2C). 268 Analysis with acetate concentrations showed no correlation with IDO-1 expression (Figure 269 2D).

270 We validated experimentally the observed correlations by testing the effect of a range 271 of physiological intestinal concentration of SCFAs on *IDO-1* reporter system. Acetate which 272 is the more abundant SCFA produced by gut bacteria had no impact on IDO-1 expression. 273 Butyrate and to a lesser extent propionate, isobutyrate, isovalerate and valerate down-274 regulated IDO-1 (Figure 3A and Supplementary Figure 2B). Indeed, as shown in Figure 3A, a 275 significant IDO-1 down-regulation was observed at a concentration as low as 0.5 mM for 276 butyrate and propionate. These concentrations were consistent with the final SCFA 277 concentrations in bacterial supernatants used in the screen thus supporting their involvement 278 in *IDO-1* down-regulation (Supplementary Table 1). Butyrate and propionate are found in the 279 human gut lumen at around 20mM (Cummings et al., 1987). Moreover, we showed that butyrate and propionate also inhibited Interferon  $\gamma$  (IFN $\gamma$ )-induced *IDO-1* expression in a 280 281 dose-dependent manner in our reporter system (Figure 3B and Supplementary Figure 2C). 282 This result was confirmed at the mRNA level by RT-PCR in IFNy-treated HT-29 cells with a 283 total abolishment of IDO-1 expression by butyrate and propionate while acetate had no 284 significant impact (Figure 3C). In addition, the inhibitory impact of butyrate and propionate 285 on IDO-1 expression was observed in an IDO-1 reporter system expressed in another IEC 286 line, Caco-2 (Figure 3D and Supplementary Figure 2D). More importantly, we showed that 287 this phenotype is not restricted to cell-lines as IDO-1 mRNA level was also significantly 288 down-regulated by butyrate in human primary colonocytes culture, compared to non-treated 289 cells (Figure 3E).

### 290 Butyrate inhibits IFNγ-induced *IDO-1* expression by STAT1 down-regulation

Several mechanisms of *IDO-1* induction have been reported. A classical cascade
 involves IFNγ-dependent phosphorylation of Signal transducer and activator of transcription 1
 (STAT1) promoting *IDO-1* expression (Chon et al., 1996). Previous studies have

294 demonstrated the inhibition of IFNy-dependent phosphorylation of STAT1 by butyrate, in a 295 nasopharyngeal carcinoma model (Jiang et al., 2010; He et al., 2013). We thus assayed by 296 immunoblot analysis the impact of a 24h-treatment of butyrate on the IFNy-induced 297 phosphorylation of STAT1 in HT-29 cells. In line with other studies, we observed less Tyr 298 701 phosphorylated form of STAT1 in cells pre-treated with butyrate (Figure 4A-B). 299 Interestingly, in contrast to previous studies, we observed that this phenotype was directly 300 correlated to a down-regulation of the protein level of STAT1 itself mediated by butyrate as 301 both total STAT1 and phosphorylated STAT1 levels were similarly diminished (Figure 4A-302 C). The butyrate-driven STAT1 down-regulation was observed on both IFNy stimulated and 303 non-stimulated cells (Figure 4A, 4C and Supplementary Figure 3A). Interestingly, we did not 304 monitor any inhibition of STAT1 gene expression by RT-PCR at 6 and 24h post incubation 305 with butyrate (Supplementary Figure 3B) suggesting post-transcriptional modifications of 306 STAT1. To further determine whether STAT1 was translocated in the nucleus by butyrate 307 treatment, nuclear STAT1 protein level was assessed by immunoblotting in butyrate-treated 308 and control HT-29 cells. As shown in figure 4D, we did not detect accumulation of nuclear 309 STAT1 in butyrate-treated cells. In summary, these findings demonstrated that butyrate 310 strongly reduced STAT1 protein level which is a mechanism contributing to the inhibition of 311 IFNy-induced *IDO-1* in human intestinal epithelial cells.

# 312 Butyrate inhibits *IDO-1* expression independently of STAT1 and STAT3.

To further decipher the mechanism of butyrate-driven *IDO-1* regulation observed in cells untreated with IFNγ, we studied STAT1 involvement in the *IDO-1* down-regulation observed in unstimulated IECs (Figure 3). The pivotal role of STAT1 was assayed using siRNA down-regulation (Supplementary Figure 4A). We observed no impact on butyratedependent inhibition of *IDO-1* in absence of STAT1 signalling. These results suggested that butyrate did not impact on basal STAT1-dependent signalling and that STAT1-independent
mechanism may also be involved in *IDO-1* down-regulation (Figure 5A).

320 Two alternative pathways for *IDO-1* induction have been reported, involving STAT3 and arvl hydroxycarbon receptor (AHR) on one hand and an NFkB-dependent pathway on the 321 322 other hand (Litzenburger et al., 2014; Yu et al., 2014; Li et al., 2016). We showed that 323 blocking STAT3 phosphorylation (Cucurbitacin I) or activating AHR pathway (TCDD) did 324 not induce IDO-1 or prevent butyrate inhibition in our model supporting that the 325 STAT3/AHR pathway was not involved in this process (Figure 5B-C). Moreover, 326 immunoblotting assays on STAT3 level revealed, that in contrast to STAT1, STAT3 was not decreased following butyrate incubation for 24h in HT-29 (Supplementary Figure 4B). In 327 328 addition, we ruled out NFkB activation as NFkB inhibitor BAY 11-7082 did not impact on butyrate-driven *IDO-1* down-regulation, as positive control NFkB activation was induced by 329 TNFα (Figure 5D). Altogether, these results suggested that butyrate down-regulated *IDO-1* 330 331 independently of STAT1, STAT3, AHR and NFkB.

# Butyrate-mediated impact on *IDO-1* is independent of the SCFAs receptors GPR41, GPR43 and GPR109a.

334 Our data suggest that butyrate down-regulates IDO-1 expression in a STAT1 and 335 STAT3-independent manner and, thus, might involve an additional mechanism. SCFAs 336 impact human cells through two main mechanisms: inhibition of histone and lysine 337 deacetylases (K/HDAC) and activation of specific G-protein coupled receptors (GPR41, 338 GPR109a: both Ga/i coupled receptors and GPR43: Ga/i and Gaq coupled receptor) 339 (Hinnebusch et al., 2002; Tolhurst et al., 2012; Schilderink et al., 2013). We confirmed that 340 the three G-protein coupled receptors are expressed in HT-29 and Caco-2 cells 341 (Supplementary Figure 5A-B). To test the potential role of these receptors, we first used

selective agonists of GPR41 (1-MCPC and AR420626), GPR43 (Tiglic acid and 4-CMTB) 342 and GPR109a (Niacin and MK1903). If the butyrate-driven down-regulation of IDO-1 343 344 expression were mediated by the GPR-dependent signalling pathways, we should expect that 345 activation of these receptors would inhibit IDO-1 expression. Interestingly, none of these 346 agonists, alone or in combination, impacted *IDO-1* expression (Figure 6A and Supplementary 347 Figure 5C). To further confirm this observation, we used inhibitors of the Gai and the Gaq 348 pathways: the pertussis toxin (Ptx) and phospholipase Cβ inhibitor (U73122) respectively. As 349 shown in Figure 6B, none of these inhibitors impacted on the butyrate-dependent IDO-1 350 down-regulation. Moreover, over-expression of GPR43 and GPR109a in HT-29 did not 351 impact the butyrate-dependent inhibition of IDO-1 expression (Supplementary Figure 6). 352 Altogether these results suggest that the SCFAs receptors GPR41, GPR43 and GPR109a were 353 not involved in the observed butyrate-driven inhibition of IDO-1 expression.

# Butyrate down-regulates *IDO-1* expression *via* its HDAC inhibitory property in a AP-1, PPARγ and SP1-independent manner

356 SCFAs, and butyrate in particular, are potent modulators of protein acetylation targeting 357 histones and transcription factors. Indeed, SCFAs impact human cells through their ability to 358 inhibit lysine and histone deacetylases (HDAC) and are thus considered as members of the 359 HDAC inhibitor (HDACi) family (Schilderink et al., 2013; Koh et al., 2016). As part of the 360 aliphatic family of HDACi, butyrate targets HDAC class I (HDAC 1, 2, 3, 8) and IIa (HDAC 361 4, 5, 7, 9) (Gallinari et al., 2007). To assess if butyrate impactes *IDO-1* expression through its 362 HDACi property, we tested three HDACi targeting a wide range of HDAC. Two belonging to 363 the hydroxamic acids family, structurally and metabolically unrelated to SCFAs: trichostatin 364 A (TSA), Vorinostat (SAHA) and one belonging to the fatty acid family: sodium valproate 365 (VAP) (Gallinari et al., 2007). The effect of butyrate on *IDO-1* expression was mimicked by

the three HDACi tested suggesting that the *IDO-1* down-regulation observed with butyratemight be a consequence of its HDAC inhibitory properties (Figure 7A).

368 Regulation of gene transcription by butyrate involved a wide range of transcription 369 factors. To delineate whether transcription factors targeted by butyrate could impact IDO-1 370 expression, we analysed the human IDO-1 promoter sequence. Analysis revealed binding 371 sites for several transcription factors implicated in butyrate-regulated gene expression, namely 372 Specificity Protein-1 (SP1) binding GC-rich boxes, as well as AP1 and PPARy responsive 373 elements (Supplementary Table 2) (Nakano et al., 1997; Davie, 2003; Nepelska et al., 2012; 374 Alex et al., 2013). To delineate if butyrate affects IDO-1 expression via SP1, we treated 375 stimulated cells with mithramycin A that binds to GC-rich DNA sequences, thereby inhibiting 376 SP1-dependent gene modulation (Blume et al., 1991). As shown in Figure 7A, incubation of 377 butyrate or HDACi-stimulated cells with mithramycin did not impact on the IDO-1 down-378 regulation, suggesting that SP1 was not involved in this process. As butyrate is a major 379 activator of PPARy-dependent gene activation, we also investigated its role in IDO-1 down-380 regulation (Alex et al., 2013). Two specific PPARy activators, pioglitazone and rosiglitazone, 381 did not affect IDO-1 expression, suggesting that the PPARy responsive elements in IDO-1 382 promoter might not be functional (Figure 7B). We further tested whether PPARy was 383 involved in the butyrate-dependent inhibition of IDO-1 by using a specific PPAR $\gamma$  inhibitor 384 (GW9662). The PPARy inhibitor GW9662 did not impact on the butyrate-induced IDO-1 385 down-regulation, confirming that the transcription factor PPAR $\gamma$  was not involved in this 386 process (Figure 7B). Finally, the implication of AP1 motifs, present in *IDO-1* promoter was 387 tested using an AP1 chemical inhibitor (SR11302). Pre-treatment with AP1 inhibitor did not 388 significantly prevent the inhibition of *IDO-1* mediated by butyrate, suggesting that AP1 was 389 not involved either (Figure 7C). Altogether, our findings suggest that butyrate down-regulates

- *IDO-1*-expression by a second mechanism involving its iHDAC property, independently of
- 391 the butyrate-targeted transcription factors AP1, PPARγ and SP1.



#### 394 **Discussion**

395 The immune system is traditionally viewed as a highly elaborated defence system developed 396 to fight intruders, especially rapidly evolving pathogens such as bacteria. However, 397 accumulating studies highlight a widespread cooperation established between hosts and 398 bacteria during millions of years that have shaped their own development (Smith et al., 2007). 399 Intestinal commensal bacteria are crucial for the development and maintenance of a healthy 400 immune system locally and have a homeostatic role beyond the gut, therefore contributing to 401 the global wellbeing of their host. The particular abundance and combination of commensal 402 bacteria may have dramatic and specific impacts on the host immune system through their 403 intimate interaction with the host epithelium. Accordingly, the intestinal epithelial cells (IEC) 404 play a central role in the dialogue established between the host and the microbiota by 405 providing an active physical segregation of commensal bacteria and by initiating the first 406 microbial-dependent signals. Indeed, IECs express receptors recognising microbial motifs that 407 activate downstream signalling cascades thus promoting the production of bactericidal 408 peptides and the recruitment and activation of innate and adaptive immune cells notably by 409 the production of effector proteins and enzymes (Atarashi et al., 2011; Kagnoff, 2014). 410 Amongst the effector molecules modulating the immune responses produced by IECs, 411 indoleamine 2,3-dioxygenase-1 (IDO-1) has an important role in the gut homeostasis 412 (Cheravil, 2009; Ciorba, 2013). However, whether human IECs express IDO-1 and how 413 bacteria control IDO-1 expression in IECs is still unclear. Here, we show that human normal 414 colonic IECs express IDO-1 at the mRNA and protein level and that epithelial IDO-1 is 415 modulated by short chain fatty acids (SCFAs), more specifically by butyrate. Indeed, we 416 demonstrate herein that physiological concentrations of butyrate down-regulate IDO-1 417 expression in HT-29 and Caco-2 reporter systems, but also at the mRNAs level in both the 418 HT-29 cell line and in human primary colonic epithelial cells (Cummings et al., 1987).

419 In the context of IFNy stimulation, STAT1 is an essential mediator of IDO-1 expression (Chon et al., 1996). Our results indicate that butyrate-treated IECs showed reduced 420 421 STAT1 phosphorylation on the tyrosine 701, as described in other models (Jiang et al., 2010; 422 He et al., 2013). However, our results indicate that the reduced amount of phosphorylated 423 STAT1 observed with butyrate is a consequence of a butyrate-driven STAT1 protein level 424 reduction. STAT1 diminution was not a result of an increase of nuclear translocation and we 425 did not observe any transcriptional inhibition of STAT1 expression, suggesting a post-426 transcriptional modification of STAT1. Many post-translational modifications of STAT1 such 427 as SUMOvlation and ubiquitination have been identified leading to STAT1 degradation and 428 consequently modifying STAT1 protein levels in cells (Tanaka et al., 2005; Yuan et al., 2012; 429 Maarifi et al., 2015). Interestingly, butyrate has been described as a global enhancer of protein 430 ubiquitination (Jiang et al., 2010). We thus believe that combination of post-translational 431 modifications of STAT1 might occur explaining its down-regulation by butyrate. The precise 432 mechanism, and cellular actor, notably the implication of HDAC inhibition or GPRs 433 implicated in STAT1 down-regulation need to be investigated further.

434 In addition to the butyrate-dependent down-regulation of STAT1 that impaired IFNy-435 induced IDO-1 expression, we demonstrated that STAT1 is dispensable for the basal IDO-1 436 repression induced by butyrate suggesting that this SCFA repressed IDO-1 expression by a 437 second distinct mechanism. To decipher this STAT1-independent mechanism, we 438 investigated the implication of butyrate specific G-protein coupled receptors (GPR41, 439 GPR109a and GPR43). However, by using agonists of these receptors and G protein subunit 440 inhibitors, we showed that this mechanism was not implicated in the inhibition of IDO-1 441 mediated by butyrate. SCFAs impact the host biological responses by the direct regulation of 442 gene transcription by their properties of lysine deacetylase inhibitors that consequently favour 443 acetylation of histones and transcription factors (Hinnebusch et al., 2002; Schilderink et al.,

444 2013). We showed that three HDAC inhibitors targeting a wide range of HDAC mimicked the 445 effect of butyrate on IDO-1 expression in un-stimulated cells suggesting that the IDO-1 446 down-regulation observed was likely linked to the HDAC inhibitory properties of SCFAs. As 447 regulation of gene transcription by HDACi involved many transcription factors, we reported, 448 by analysing the sequence of the *IDO-1* promoter, the presence of responsive elements of 449 three transcription factors potentially targeted by butyrate: SP1, AP1 and PPARy (Nakano et 450 al., 1997; Davie, 2003; Nepelska et al., 2012; Alex et al., 2013). However, by using specific 451 inhibitors and agonists, we demonstrated that these three transcription factors were not 452 involved in the STAT1-independent butyrate-driven inhibition of IDO-1 expression.

453 Despite being limited to human cell-lines and primary IECs, our results highlighted a role of butyrate in IDO-1 expression. However in vivo studies are required to confirm these in 454 455 vitro results and to precise the downstream effects of modulation of *IDO-1* in the colon. What 456 would be the impact of IDO-1 inhibition on human health is still an open question, as, 457 depending on the disease context, its expression has positive or negative outcomes (Fallarino 458 et al., 2012; Metghalchi et al., 2015; Laurans et al., 2018; Metghalchi et al., 2018). IDO-1 is 459 highly expressed in human tumour cells and consequently creates an immunosuppressive 460 microenvironment that has been associated with poor prognosis notably in colorectal cancer 461 (Cheravil, 2009; Ciorba, 2013). IDO-1 expression is high in inflammatory bowel diseases 462 notably in IECs and has often been positively associated with the severity of gastrointestinal 463 diseases and inflammatory-induced colon tumorigenesis, with no causal implication (Barcelo-464 Batllori et al., 2002; Ferdinande et al., 2008; Cheravil, 2009; Ciorba, 2013). However IDO-1<sup>-/-</sup> 465 mice do not present any spontaneous colitis and its role in induced colitis models varies 466 between studies according to the inducing agent and mouse strain used and probably the 467 microbiota composition (Gurtner et al., 2003; Ciorba et al., 2010; Takamatsu et al., 2013; 468 Shon et al., 2015). IDO-1 regulates immune responses via the so-called "metabolic immune

469 regulation" that suppresses the Th1 and Th17 differentiation and enhances the de novo differentiation of anti-inflammatory regulatory T cells (Fallarino et al., 2012). A recent study 470 471 suggests that the role of IDO-1 in the regulation of the immune response is more complex as 472 it repressed the production of IL10, a major anti-inflammatory cytokine (Metghalchi et al., 473 2015). In line with this, recent studies suggest that IDO-1 expression have a detrimental role 474 in aneurysm, atherosclerosis and obesity (Metghalchi et al., 2015; Laurans et al., 2018; 475 Metghalchi et al., 2018). Moreover, Laurans et al. demonstrate that IDO-1 activity enhanced 476 chronic inflammation and intestinal permeability that consequently impacts on obesity 477 outcomes (Laurans et al., 2018). In addition, IDO-1 has been described as a main regulator of 478 the intestinal B cell responses to commensal bacteria that drives microbiota composition and 479 indirectly the microbiota-dependent barrier responses (Harrington et al., 2008; Zelante et al., 480 2013). These studies demonstrate that intestinal IDO-1 expression might also shape gut 481 microbiota with potent impact on host health. Altogether, these studies suggest that the role of 482 IDO-1 in influencing gut inflammation is far more complex than expected, and might depend 483 on the cell types expressing it. IDO-1 down-regulation by microbiota-derived butyrate in 484 IECs, as demonstrated here, could be crucial for the fine-tuning of IDO-1 expression in 485 healthy conditions and for the initiation of appropriate immune responses depending on the 486 context: chronic inflammation, cancer, obesity or infections.

Here, we describe an important role for the SCFA butyrate in the regulation of *IDO-1* expression in IECs. Contrary to DCs where IDO-1 functions in diverse processes in health and disease have been well documented, its role in IECs is still debated. We demonstrated here for the first time that butyrate represses *IDO-1* expression by two distinct mechanisms. First, butyrate treatment was able to reduce STAT1-dependent induction of *IDO-1*. In addition, we show that this reduction is correlated with the butyrate-driven decrease in STAT1 level. Second, butyrate regulation of *IDO-1* expression is independent of the IFNγ-

494 signalling pathway and involves the HDAC inhibitory property of butyrate. As SCFAs are
495 crucial for human physiology and health, our results strongly suggest that controlling *IDO-1*496 expression in IECs under steady state conditions can be part of the global mechanism of
497 SCFAs to maintain immune homeostasis in the gut.



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### 511 Authors Contribution

512 Conceived and designed the experiments: CMG, NL; performed most of the experiments:

513 CMG; performed some experiments: PL, AJ, FBC, LM, FL, NL; analysed the data: CMG, AJ,

- 514 NL; contributed materials: FR; wrote the paper: CMG, NL; edited and revised the manuscript:
- 515 AJ, PL, FR, HMB.
- 516

#### 517 **Disclosures:**

- 518 The authors disclose no conflict of interest.
- 519
- 520

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#### 708 Figure legends

#### 709 **Figure 1:**

710 IDO-1 expression in human colonic epithelial cells. (A) Human normal colonic mucosa was 711 stained for IDO-1. Representative immunohistochemical staining of IDO-1 showed that IDO-712 1 (brown) is expressed in epithelial cells (left panel: strong perinuclear and/or membrane 713 staining of about 80% of the IECs ; right panel: heterogeneous staining of few IECs (arrows)) 714 and in few lamina propria mononuclear cells (arrowheads) and endothelial cells (asterisk) 715 (original magnification x200). (B). IDO-1 gene expression was determined by RT-PCR on 716 RNA extracted from preparations of isolated human colonic epithelial cells (IECs) and of whole mucosa microdissected from normal colon. Results were normalised to  $\beta$ -2 717 *microglobulin (B2M)* and expressed as 2- $\Delta$ Ct relative value (median ± quartiles) of 4 patients 718 719 (1-2 samples/patient).

### 720 Figure 2:

721 Correlation between bacterial metabolites production and *IDO-1* gene expression. (A) Effect 722 of bacterial supernatants on *IDO-1* reporter system organised by phylum. Culture supernatants 723 of a wide range of cultivable commensal bacteria were applied on the HT-29-IDO-1 reporter 724 system (10% vol/vol) for 24h. IDO-1 expression was measured by luciferase activity and 725 expressed as fold increase towards its control: non inoculated growth medium used for each 726 culture. IDO-1 expression profiles upper and lower the dash lines were considered as significantly changed. (B) PCA analysis showing the correlation between the SCFAs 727 728 concentrations produced by the commensal bacteria and IDO-1 expression. (C). Representation of *IDO-1* expression correlated to butyrate concentration in bacterial cultures 729

classified by rank value. Actinobacteria in blue, Bacteroidetes in yellow, Firmicutes in grey,
Fusobacteria in red and Verrucomicrobiea in light blue.

732 **Figure 3**:

733 Impact of SCFAs on *IDO-1* expression. (A), HT-29-*IDO-1* reporter cells were incubated with a range of concentration of acetate, butyrate and propionate (0.5; 1; 2; 4; 8 mM) for 24h. IDO-734 735 *l* expression was measured by luciferase activity and expressed as the mean  $\pm$  SD fold change 736 towards un-stimulated cells (N>3). (B), HT-29-IDO-1 reporter cells were incubated with IFNy (100U/ml) and a range of concentration of butyrate (0.5-8mM). IDO-1 expression was 737 738 measured by luciferase activity and expressed as the median  $\pm$  quartiles of fold change 739 towards un-stimulated cells (N>3). (C) IDO-1 gene expression on HT-29 exposed for 6h to 740 IFNγ (100U/ml) +/- butyrate (2mM), propionate (4mM) or acetate (8mM) was determined by 741 RT-PCR. Results were normalised to *GAPDH* and expressed as  $2-\Delta\Delta$ Ct relative to control 742 mean value; ND: not detected (N=3). (D) Caco2-IDO-1 reporter cells were incubated with a 743 range of concentration of acetate, propionate and butyrate (0.5; 1; 2; 4; 8 mM). IDO-1 744 expression was measured by luciferase activity and expressed as the mean  $\pm$  SD fold change 745 towards un-stimulated cells (N>3). (E) IDO-1 expression level on human colonic epithelial 746 cells treated for 24h with butyrate compared to non-treated cells from the same patient was 747 determined by RT-PCR. Results are normalised to *RPS17* and expressed as 2- $\Delta\Delta$ Ct relative to 748 control, median  $\pm$  quartiles (N=4). P value: \*P<0.05, \*\*P<0.005, \*\*\*P<0.001.

749 **Figure 4**:

Inhibition of IFN $\gamma$ -induced *IDO-1* expression by butyrate is correlated with a decrease of STAT1 protein level. (A-C) HT-29 cells were cultured 24h with butyrate (But 2mM) prior IFN $\gamma$  (100U/ml) stimulation for 15 (line 3 with butyrate and 5 without butyrate) or 30 min

753 (line 4 with butyrate and 6 without butyrate). The protein level of p-STAT1 Tyr701, STAT1 754 and Actin were determined by western-blot on total protein extracted. Densitometric 755 quantifications of total P-STAT1 and STAT1 proteins, from 3 independent experiments, were 756 normalised to Actin and expressed as fold change compared to IFN stimulated cells (B) and 757 unstimulated cell (C) respectively of 3 independent experiments. Data are represented as 758 median  $\pm$  quartiles. (D) HT-29 cells were incubated 24h with medium or butyrate (But 2mM) 759 prior cytoplasmic and nuclear extractions. The protein levels of STAT1, Laminin A/C and 760 GAPDH were assessed in each fraction by western-blot. P value: \*P<0.05, \*\*P<0.005, 761 \*\*\*P<0.001.

#### 762 **Figure 5**:

Butyrate inhibition of IDO-1 promoter activity is STAT1 and STAT3 independent. (A) HT-763 29-IDO-1 cells were transfected with STAT1 siRNA or control siRNA and incubated with 764 765 butyrate (But 2mM) or IFNy (100U/ml) for 24h before measuring IDO-1 level. (B) HT-29-766 *IDO-1* cells were incubated for 2h with the STAT3 phosphorylation inhibitor (Cucurbitacin I, 767 1µM) prior to butyrate (But 2mM) treatment for total incubation time of 24h (N=4). (C) HT-768 29-IDO-1 cells were incubated with AHR ligand (TCDD 10nM) +/- butyrate (But 2mM) for 769 24h. Data represented 2 independent experiments (D) HT-29-IDO-1 cells were incubated for 770 1h with the NKFB inhibitor, Bay117082 (Bay 40µM) prior stimulation with butvrate (But 771 2mM) or TNFa (10ng/ml) for 24h (N=3). IDO-1 expression was measured by luciferase activity and expressed as median  $\pm$  quartiles of fold change towards unstimulated cells. Data 772 773 represented at least 3 independent experiments. P value: \*P<0.05, \*\*P<0.005, \*\*\*P<0.001.

#### 774 **Figure 6:**

775 Butyrate mediated impact on IDO-1 is independent of its receptors GPR41, GPR43 and 776 GPR109a. (A) HT-29-IDO-1 reporter cells were incubated for 24h with selective GPR 777 agonists: GPR41: AR420626 (1µM) and 1-MCPC (1mM); GPR43: 4-CMTB (1µM) and 778 Tiglic acid (1mM); GPR109a: Niacin (1mM) and MK1903 (1µM) or with DMSO (vehicle), butyrate (But 2mM) or Control (RPMI). (B) HT-29-IDO-1 reporter cells were incubated for 779 780 24h with 2mM butyrate +/- GPRs sub-unit inhibitors: Pertussis toxin (Ptx, 0.2µg/ml), U73122 781 (10µM) or glycerol (vehicle). IDO-1 expression was measured by luciferase activity and 782 expressed as median  $\pm$  quartiles of fold change towards un-stimulated cells. Data represented 783 at least 3 independent experiments. P value: \*P<0.05, \*\*P<0.005, \*\*\*P<0.001.

## 784 **Figure 7:**

785 HDAC inhibitor mimicked the butyrate-dependent down-regulation of IDO-1 expression in a 786 SP1, PPARy and AP-1 independent manner. (A). HT-29-IDO-1 reporter cells were incubated 787 for 24h with butyrate (But 2mM), SAHA (5µM), Trichostatin A (TSA 1µM) or Valproic acid 788 (VAP 5mM)  $\pm$  SP1 inhibitor (Mitramycin A; MitA 0.1µM). (B), HT-29-*IDO-1* reporter cells 789 were stimulated for 24h with two PPAR $\gamma$  activators: Pioglitazone (Pio 5 $\mu$ M); Rosiglitazone 790 (Rosi,  $10\mu$ M) or the specific PPAR $\gamma$  inhibitor GW9662 ( $10\mu$ M) ± butyrate (But 2mM). (C) 791 HT-29-IDO-1 reporter cells were incubated for 24h with butyrate (But 2mM) and/or the AP1 792 inhibitor, SR11302 (10µM). IDO-1 expression was measured by luciferase activity and 793 expressed as median  $\pm$  quartiles of fold change towards un-stimulated cells. Data represented 794 at least 3 independent experiments. P value: \*P<0.05, \*\*P<0.005, \*\*\*P<0.001.



HC'S

Total mucosa

Martin-Gallausiaux *et al.* Figure 1





Figure 4.TIFF







Martin-Gallausiaux *et al.* Figure 6



Martin-Gallausiaux *et al.* Figure 7