In vitro assessment of the effect of lactose-free milk on colon microbiota of lactose intolerant adults

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Running title: *In vitro* colon model of lactose intolerant adults

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Abstract
Milk is an essential food, but a large part of adult's population is uncapable to digest lactose. Lactose intolerance can seriously affect the intestinal ecology and compromise host's wellbeing. In this scenario, the role of human gut microbiota is crucial, but little is known on that because few are the research studies conducted, either via clinical trials or via *in vitro* models. Due also to the call to reduce animal testing in science, an *in vitro* model with gut microbiota of lactose-intolerant adults is necessary. This paper wants to propose an *in vitro* model coupling oro-gastro-duodenal digestion to colonic fermentation to evaluate lactose impact on colon microbiota of lactose-intolerant adults. Microbiomics and metabolomics in respect to a baseline of fermentation were compared. Generally, when the insult was given, taxa specialized for dairy sugars were unaffected, but *Bacteroidaceae* and *Lachnospiraceae* were underrepresented. Lactose triggered raise to opportunistic *Proteobacteria*, dominated by harmful *Klebsiella*. Also, an important reduction of essential short chain fatty acids was observed, and in particular that of butyrate. Although, more observations need to be conducted, as well as a comparison with the healthy condition, the present work gives results for pre-clinical application in the sight to reduce animal testing.

Keywords: MICODE, Indole, Butyrate, Volatilome, *Clostridiales*, Human Colon Microbiota

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Introduction

More than half of world's population is unable to digest lactose (Campbell et al., 2005), due to lack or low expression in endogenous beta glucosidase and follow-on in maldigestion or intolerance that trigger gut inflammation worsening life condition. In order to treat or mitigate such disorders, the safest way is that of avoiding intake of any product containing lactose, because few are the alternative solutions paved. The etiology is still far to be defined, because up to date research focused on aspects not considering the human gut microbiota, while this feature has a central role due to the contribution of bacterial beta-glucosidases. Given such scenario, future research must consider elucidating the impact of lactose on gut microbiota.
At present, few clinical studies investigated the effect of modulation of gut microbiota in lactose-intolerant adults applying probiotics administration (Cano-Contreras et al, 2022; Vitellio et al., 2019; Pakdaman et al, 2015; He et al, 2008), but the number of different probiotics species and their combination make a systematic study in humans almost impossible. Although clinical studies remain the gold standard, a valid tool would be needed to pre-screen the most effective species/combinations to be then tested in humans. Mice and pigs models (Xue et al., 2020; Alexandre et al., 2013) are not a valid tool since they have a different microbiota, and this makes difficult to translate results to humans. Even in humanized animals (Ntemiri et al. 2019), the model is based on microbiota from elderly, which does not reflect that of an adult (Kim & Jazwinski 2018). In addition, science is moving towards a drastic reduction in animal experimentation.
In this light, the development of suitable *in vitro* models is increasingly necessary. In literature, the few studies on lactose intolerance carried out using *in vitro* models used human colon microbiota (HCM) from healthy donors (Makivuokko et al., 2006; Windey et al., 2015), whose composition may be deeply different from that of lactose intolerants. Moreover, most of the aforementioned studies used pure compounds (lactose) and not food matrices (milk or dairy products).
Here, we propose an *in vitro* model for gastric digestion and colonic fermentation based on the HCM of lactose-intolerant adults, in order to assess the impact generated after intake of milk with and without lactose. We combined the INFOGEST digestion protocol (Minekus et al., 2014), realized within the INFOGEST COST action (2011-2015), to colon fermentation with MICODE model (Multi Unit *In vitro* Colon Model) (Nissen et al., 2021; Nissen et al., 2021a; Nissen et al., 2022; Nissen et al., 2022a) to study the perturbations in microbiota composition and microbial metabolites production and obtain data with preclinical robustness.

Materials and Methods

Human Colon Microbiota

HCM was obtained from the stools of two lactose-intolerant volunteers. The volunteers were adults with positivity to lactose breath test, not consuming antibiotics, pre- or probiotic supplements in the 3 months prior to the experiment, normal weight, non-smokers, and with no history of chronic gastrointestinal disorders. Volunteers were informed of the purposes and procedures of the study and provided their written informed consent, in accordance with the Ethics Committee of the University of Bologna.

Human stools were collected by volunteers in a dedicated sterile container, placed in an anaerobic jar with oxygen catalyst (Oxoid, Thermo Fisher Scientific, USA), transferred to the laboratory, and processed within 2 h. HCM was obtained by homogenizing 2 g of each donation in 36 mL of pre-reduced phosphate buffered saline (Wang et al, 2020; Nissen et al, 2022).

Materials

Reagents for *in vitro* digestion and colonic fermentation were purchased from Sigma-Aldrich (St. Louis,MO, USA) and Carlo Erba Reagents (CEDEX, Val de Reuil, FR), unless otherwise stated. Reagents for molecular biology (PCR and qPCR), kits for DNA extraction and genetic standard purifications, were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

***In Vitro* Digestion and Fermentation**

UHT semi-skimmed milk (L) and UHT semi-skimmed lactose-free milk (LF) were purchased from Granarolo S.p.A. (Bologna, Italy). Milks were digested *in vitro* following the INFOGEST protocol (Minekus et al., 2014) realized within the INFOGEST COST action (2011-2015), and the digestates were then stored at -80 °C. Briefly, the digestion process was performed on 5 mL of milk for 242 min (2 min of oral, 120 min of gastric and 120 min of intestinal digestion) at 37 °C. During *in vitro* digestion, consecutive enzymatic treatments were performed by the addition of simulated saliva (containing 75 U/mL alpha-amylase), simulated gastric juice (2000 U/mL pepsin) at pH 3, and simulated pancreatic juice (10mM bile and 100 U/mL pancreatin) at pH 7. After digestion, the resulting solutions were frozen at -80 °C until further *in vitro* colonic fermentation.

Prior to *in vitro* colonic fermentation, the digestates were thawed and gently centrifuged to precipitate the denser portion and 1 mL of that was then applied in MICODE bioreactors.

Briefly, short-term batch proximal colon fermentations were conducted for 24 hours in independent bioreactors using the *in vitro* colon model, MICODE (Nissen et al., 2021; 2021a; 2022; 2022a). The preparation of the experiments was made according to published procedures (Connolly et al., 2012; Koutsos et al., 2017; Wang et al., 2020) and described in detail in Nissen et al. (2021; 2021a; 2022). Concisely, bioreactors were autoclaved at 121 °C and -1 bar for 15 min and once cooled, aseptically filled with 90 mL of anaerobic pre-sterilized basal nutrient medium.

Basal medium (Connoly et al., 2012; Diotallevi et al., 2021) contained (per L): 2 g peptone, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4\_7H_2O$, 0.01 g $CaCl_2\_6H_2O$, 2 g $NaHCO_3$, 2 mL Tween 80, 0.05 g Hemin dissolved in 1 mL of 4 M-NaOH, 10 mL vitamin K, 0.5 g L-cysteine HCl, and 0.5 g bile salts (sodium glycocholate and sodium taurocholate). The medium was adjusted to pH 7.0 before autoclaving and 2 mL of 0.025% (w/v) resazurin solution were added afterwards.

Bioreactors were left running to reach and maintain the proximal colon ecological conditions (0.0% of DO_2, pH 5.75, 37 °C of temperature, and 300 rpm of stirring), by constant flushing with filtered O_2-free N_2, Peltier heater, and automatic addition of filtered NaOH or HCI (0.5 M),

Afterwards, the three different bioreactors were aseptically loaded with 9 mL of fecal slurry (10% w/v of human feces in O_2 reduced PBS) and: i) 1 mL of digestated LF; ii) 1 mL of digestated L; or iii) 1 mL of deactivated digestive enzymes as the blank control (BC). After adaptation to the ecological conditions, considered as the baseline (BL) corresponding to 1.52 ± 0.18 h, when for the first time the pH changes (Applisense, Applikon Biotechnology BV, NL), the batch cultures were run under controlled conditions and 4 mL were sampled at different time points (BL; intermediate point (T1) = 18 h; end point (EP) = 24 h) (Nissen et al. 2021a; Nissen et al. 2022). Sampling was performed with a dedicated double-syringe-filtered system connected to a float drawing from the bottom of the vessels without perturbing or interacting with the bioreactor's ecosystem. To guarantee a close control, monitoring and recording of fermentation parameters, the software Lucullus 3.1 (PIMS, Applikon Biotechnology BV, NL) was used. This also allowed the stability of all settings to be strictly maintained during the experiment. Fermentations were conducted in duplicate independent experiments, using a new pool of feces for each, from the same two donors.

DNA Extraction, qPCR Enumeration and 16S-rRNA Sequencing

DNA was extracted from the fecal samples and from the MICODE effluents at each time points using the Purelink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Nucleic acid purity was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK).

Enumeration of bacterial groups was made with DNA by qPCR to evidence changes in the microbiota after fermentation following previous protocols (Modesto et al., 2011; Tanner et al., 2014; Tamargo et al., 2022; Nissen et al., 2021a; Nissen et al., 2022; Nissen et al., 2022a). The changes in the abundances of 6 bacterial targets (*Eubacteria, Firmicutes, Bacteroidetes*, *Lactobacillales, Bifidobacteriaceae*, and *Enterobacteriaceae*) (Table S1) were assessed by qPCR on QuantStudio 5 System (Applied Biosystem, Thermo Fisher, Waltham, MA, USA). The shifts in abundance of qPCR values in respect to the BL were calculated as $Log_2(F/C)$ (Love et al., 2014). Technical replicas of analyses were conducted in triplicate.

Metataxonomy was conducted through16S-rRNA sequencing by IGA Technology Service Srl (Udine, Italy). Libraries were sequenced with MiSeq (Illumina Inc, San Diego, CA., USA) in paired end with 300-bp read length (Marino et al., 2019). Sequence data analysis was conducted according to previously published papers (Marino et al. 2019; Nissen et al., 2021). Technical replicas of analyses were conducted in duplicate for the BL and in pooled samples for the endpoints.

Volatilome analysis

The volatilome is the set of volatile organic compounds (VOCs) that characterizes a specific ecosystem, i.e. the human colon, and in our work represents the metabolites produced by colonic fermentation of milk samples. VOCs evaluation was performed on a Gas Chromatograph (7890A, Agilent, Santa Clara, CA, USA) Mass Spectrometer (5975, Agilent, USA) operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The SPME (Solid Phase Micro-Extraction) GC-MS protocol and the identification of volatile compounds were done accordingly to previous reports, with minor modifications (Guerzoni et al., 2007; Di Cagno et al., 2011; Nissen et al., 2020; Nissen et al., 2021; Casciano et al., 2021). Preconditioning, absorption, desorption of SPME–GC-MS analysis, and all data processing were carried out according to literature (Nissen et al., 2021; Di Cagno et al., 2011; Casciano et al., 2021). Briefly, before each head space sampling, the fiber was exposed to the GC inlet for 10 min for thermal desorption at 250 °C in a blank sample. The samples were then equilibrated for 10 min at 50 °C. The SPME fiber was exposed to each sample for 40 min and finally the fiber was inserted into the injection port of the GC for a 10 min sample desorption. The temperature program was: 50 °C for 0 min, then ramping at 1.5 °C min^{-1} to 65 °C and at 3.5 °C min^{-1} to 220 °C, which was maintained for 20 min. Injector, interface, and ion source temperatures were 250 °C, 250 °C, and 230 °C, respectively. Injections were carried out in splitless mode and helium (3 mL min^{-1}) was used as the carrier gas. Identification of molecules was carried out by searching mass spectra in the NIST 11 MSMS library (NIST, Gaithersburg, MD, USA). The VOCs were then relatively quantified (Peak Area %), sorted for respective chemical class, i.e., organic acids, alcohols, and other VOCs, and normalized (Nissen et al., 2020; Nissen et al., 2021c). In samples at BL the main microbial VOCs related to fermentation of foods were absolutely quantified in mM by SPME GC-MS with the use of an internal standard (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Nissen et al., 2021b; Di Cagno et al., 2011; Casciano et al., 2021). Changes of main microbial VOCs at T1 and EP of fermentations were evaluated in respect of the BL values. Technical replicas of analyses were conducted in duplicate.

Statistical Analysis

Normality by Shapiro Wilk's test and Homoscedasticity of Varaince by Levene's test were used for the datasets of the volatilome and the qPCR values. Multivariate ANOVA (MANOVA) model by

time and matrix categories, followed by *post hoc* Tukey HSD test ($p < 0.05$), was used to statistically analyze the datasets of volatilome and qPCR values. Principal Component Analysis (PCA) was also computed for the datasets of the volatilome. Statistic of metataxonomy was assessed following the QIIME pipeline version 2.0 (Bolyen et al., 2019). ANOVA model for time category was used for filtered OTUs (open taxonomic units). Statistic was performed with Statistica v.8.0 (Tibco, Palo Alto, CA, USA).

Results and Discussion

Volatilome Analysis

Through SPME GC-MS, 57 molecules were identified with more than 80% of similarity with NIST 11 MSMS library (NIST, USA) and presented as a quantification heatmap (Figure S1). The volatilome was subject to MANOVA model and variables selected, sorted by chemical class, and computed for PCAs.

A PCA of 11 organic acids distributed cases on the plot, separating the BL from T1 and EP of any substrates (Figure 1A). From our results, the main descriptor of fermentation with LF was Butanoic acid (MANOVA 67.17%) (Table S2). The main descriptors of L were Pentanoic, Hexanoic, and Octanoic acids, (71.78%, 65.63%, 52.40%, respectively) (Table S2), with Pentanoic and Octanoic acids mainly produced at EP (58.59% and 66.15%, respectively) (Table S3). It is known that Hexanoic acid is formed by lactose fermentation and free fatty acids lipolysis (Wang et al., 2019), in fact in LF its production was absent (Table S2).

A PCA of 11 alcohols distributed cases on the plot, separating the BL from time points of colonic fermentations and discriminating L at the EP from the others (Figure 1B).

The descriptors of L were 1-Butanol (63.36%) and Phenol (71.57%), while those of LF were Ethyl alcohol (51.07%), 1-Octanol (42.04%), and 1-Hexanol, 2-ethyl- (51.60%) (Table S2).

These molecules were manly produced at the EP of fermentation (Table S3). It is interesting to note that, according to Windey et al. (2015), a higher number of alcohols are produced during fermentation of lactose. In fact, in our study there was a higher speciation of alcohols with fermentation of L in respect to that of LF.

A PCA of 11 other VOCs distributed the cases over the plot, with poor discrimination on the basis of samples with the exception of L at the EP and BC at T1 (Figure 1C). The VOCs that defined L was 2-Hexanone (35.71%) (Table S2), while LF was described by 2-Acetylthiazole (46.54%) (Table S2) exclusively derived from the fermentation process (Table S3).

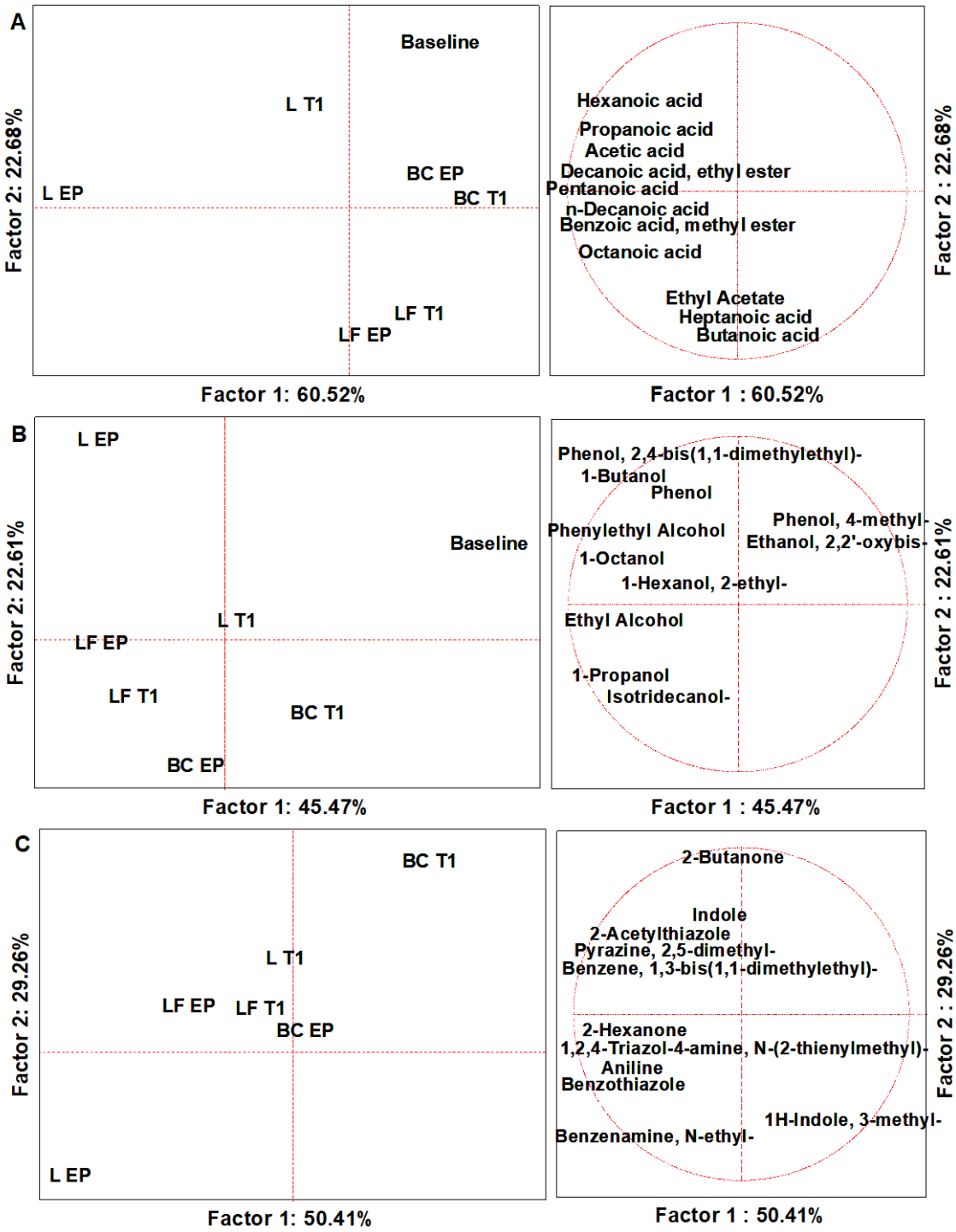

Figure 1. PCA plots of the volatilome of milk samples after colonic fermentation. Left side diagrams are for PCAs of cases; right side diagrams are for PCAs of variables. A) Acids; B) Alcohols; C) Other VOCs. BL = baseline; T1 = 18 h; EP = 24 h; L = milk with lactose; LF = milk lactose-free; BC = Blank control.

Changes in abundance of main microbial VOCs

The baseline values of quantification of Acetic acid, Propanoic acid, and Butanoic acid in our samples (Table S4) were in mM range of that recorded with similar approaches in feces of lactose intolerant adults (Windey et al., 2015). From our results in respect to BL (Figure 2A), the concentration of acetic acid increased significantly just after L fermentation that in comparison to LF was 2.18 times higher (p = 0.008120; Table S2). It is known that when lactose reaches the colon, it acts as a prebiotic and

increases the level of carbohydrate fermentation. In fact, the colonic metabolism of lactose has been reported to be associated with an increased production of SCFAs (Alexandre et al., 2013). In our dataset Butanoic acid is depleted with L fermentation, although not significantly in comparison to LF. Due that Butanoic acid is mainly produced by a healthy colon microbiota, it is clear that the lactose insult has affected those taxa butyrate-producers.

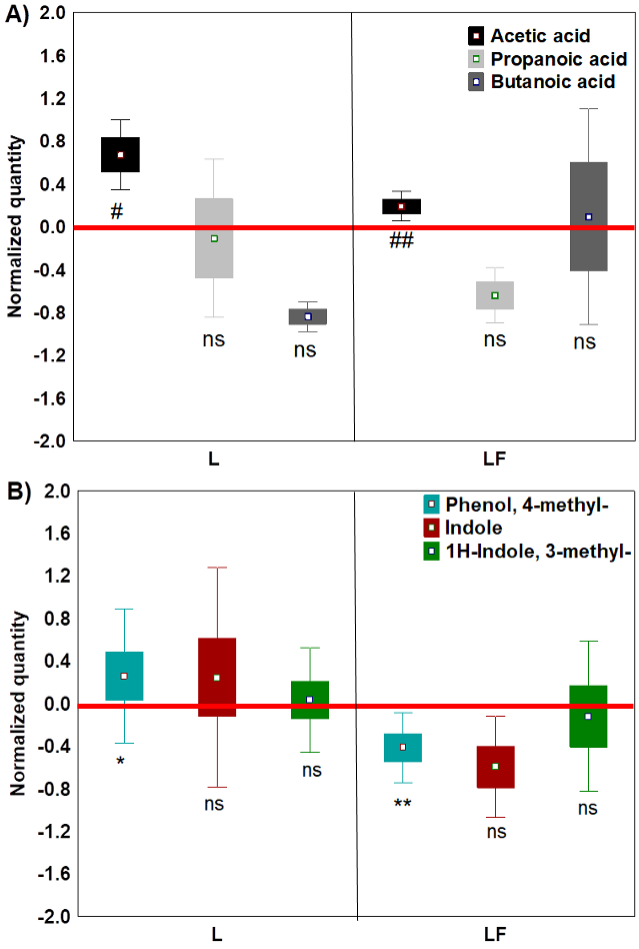

Figure 2. Changes in production of A) beneficial and B) detrimental microbial VOCs. Changes are expressed on a normalized scale in respect to the baseline of *in vitro* fermentation (red line). Box plots are including all replicas and time points. Marker = mean; box = mean ± standard error; whiskers = mean ± standard deviation. Different symbols among a single independent variable indicate significant difference according to MANOVA model followed by post hoc Tukey's HSD test. ns = not significant; L = milk with lactose; LF = milk lactose-free.

Milk is rich in aromatic amino acids, that are largely metabolized by *Proteobacteria* producing detrimental compounds for human health, as Phenol, Indole, Phenol, 4-methyl- (aka p-cresol), and 1H-Indole, 3-methyl (aka skatole) (Wang et al., 2020). Generally, p-cresol concentrations measured in human feces are rather variable (Wang et al., 2020), but our values (Table S5) were comparable with those observed by Windey et al. (2015).
From our results, fermentation of L increased and LF decreased the production of harmful VOCs, (Figure 2B). In particular, fermentation with L produced about 1.5 times more p-cresol when compared to L ($p = 0.000855$) (Table S2). This result agreed with the characterization of HCM reported below, where *Proteobacteria* increased more after L than with LF fermentations.

Changes in Abundance of Selected Bacterial Targets Absolutely Quantified by qPCR

Considering *Eubacteria*, *Firmicutes,* and *Bacteroidetes* (Table 1), fermentations of both milks decreased their abundances, with LF as the strongest. Although at EP, LF was significantly stronger than L just for *Bacteroidetes*.

Among the beneficial bacteria, at the EP both milks reduced *Bifidobacteriaceae*, while L increased and LF decreased *Lactobacillales*. Among them, lactic acid bacteria are known to be involved in lactose intolerance relief (Pakdaman et al., 2015), due to their β-galactosidase activity. In accordance, this taxon grew more with L fermentation for the presence of lactose. These results were also seen in an old *in vivo* study by Ito & Kimura (1993), where the authors showed an increase in lactobacilli and bifidobacteria after brief exposure to lactose in lactose intolerant adults.

From our results both milk samples fostered the growth of opportunistic *Enterobacteriaceae* on a time dependency.

Table 1. Changes in the absolute quantification of selected bacterial targets measured by qPCR and expressed as $Log_2(F/C)$..

qPCR Target	**Quantifications**	**Changes**		**MANOVA**
	Cells/mL ± SD	**Log_2(F/C)**		
Eubacteria	**BL**	**T1**	**EP**	
L	2.07E+09 ± 7.68E+07	0.08^{A}	-0.28	0.128684
LF	2.07E+09 ± 7.68E+07^{a}	-0.86^{bC}	-0.31^{a}	0.001363
BC	2.07E+09 ± 7.68E+07	-0.30^{B}	-0.43	0.062125
		0.000133	0.698152	*p* value
Firmicutes	**BL**	**T1**	**EP**	
L	1.60E+09 ± 8.40E+07^{b}	-1.65^{aB}	-3.10^{aB}	0.007940
LF	1.60E+09 ± 8.40E+07^{b}	-3.83^{aB}	-3.70^{aB}	0.000392
BC	1.60E+09 ± 8.40E+07	0.40^{A}	0.02^{A}	0.633917
		0.000011	0.000347	*p* value
Bacteroidetes	**BL**	**T1**	**EP**	
L	2.59E+08 ± 1.02E+07^{c}	-1.15^{aA}	-1.71^{bA}	< 0.000001
LF	2.59E+08 ± 1.02E+07^{c}	-4.06^{aC}	-4.11^{bC}	< 0.000001
BC	2.59E+08 ± 1.02E+07^{b}	-3.25^{aB}	-3.38^{aB}	< 0.000001
		< 0.000001	< 0.000001	*p* value
Lactobacillales	**BL**	**T1**	**EP**	
L	3.00E+05 ± 3.55E+04^{a}	1.13^{bB}	0.90^{cA}	< 0.000001
LF	3.00E+05 ± 3.55E+04^{a}	1.35^{bA}	-0.74^{cC}	< 0.000001
BC	3.00E+05 ± 3.55E+04^{a}	-0.30^{cC}	0.12^{bB}	0.000157
		< 0.000001	< 0.000001	*p* value
Bifidobacteriaceae	**BL**	**T1**	**EP**	
L	6.30E+05 ± 3.32E+04^{c}	-2.81^{b}	-2.34^{a}	< 0.000001
LF	6.30E+05 ± 3.32E+04^{b}	-2.06^{a}	-1.93^{a}	0.000023
BC	6.30E+05 ± 3.32E+04^{b}	-3.98^{a}	-2.59^{a}	0.000003
		0.423431	0.093098	*p* value
Enterobacteriaceae	**BL**	**T1**	**EP**	
L	7.37E+05 ± 4.39E+04^{a}	5.16^{cB}	6.14^{bC}	0.000143
LF	7.37E+05 ± 4.39E+04^{a}	8.87^{aA}	8.70^{bA}	0.000065
BC	7.37E+05 ± 4.39E+04^{a}	5.00^{aB}	7.41^{bB}	0.001236
		0.000617	0.000457	*p* value

^{A,B,C}Different capital letters indicate significance difference within a column; ^{a,b,c}Different lower case letters indicate statistical significance within a row according to MANOVA model followed by Tukey's HSD test *(P* < 0.05). MANOVA *p* values are relative to "time effect" on rows and to "matrix effect" on columns. L = milk with lactose; LF = milk lactose-free; BC = Blank control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation.

Metataxonomy of the Human Colon Microbiota Before and After *in vitro* Fermentation

Metataxonomy results of HCM demonstrated that at least two main phyla (*Bacteroidetes* and *Proteobacteria*) were significantly shifted in any samples from the BL to the EP (Table 2). Interestingly, these two are particularly involved in fibrolytic and proteolytic fermentations, respectively.

Table 2. 16S-rRNA Metataxonomy of Selected Taxa of Colonic Microbiota and Changes after 24 h of colonic fermentation*.

OTU ID[#]	**Relative Quantification (%)**				**Changes as Log_2(F/C)**			**ANOVA**	
	BL	EP			EP			*p* value	$-Log_{10}(p)$
Phylum level	mean	L	LF	BC	L	LF	BC		
Euryarchaeota	0.060	0.124	0.030	0.001	1.05	-1.01	-5.79	0.921606	0.035454
Bacteria; Other	6.752	0.059	0.285	0.127	-6.84	-4.56	-5.73	0.000413	3.384049
Actinobacteria	4.956	2.127	0.800	6.315	-1.22	-2.63	0.35	0.629394	0.201077
Bacteroidetes	35.945	0.385	1.058	9.109	-6.55	-5.09	-1.98	0.028601	1.543618
Firmicutes	49.499	22.992	55.361	25.576	-1.11	0.16	-0.95	0.548720	0.260649
Proteobacteria	2.726	74.244	42.429	58.857	4.77	3.96	4.43	0.043499	1.361520
Verrucomicrobia	0.017	0.016	0.000	0.000	-0.12	0.00	0.00	0.376325	0.424432
Family level									
Bacteroidaceae	13.241	0.196	0.742	5.814	-6.07	-4.15	-1.18	0.023573	1.627585
Bifidobacteriaceae	4.938	6.287	2.118	0.796	0.35	-1.22	-2.63	0.023010	1.638083
Enterobacteriaceae	0.625	71.875	40.229	56.971	6.84	6.00	6.50	0.095932	1.018036
Clostridiaceae	0.458	6.665	25.089	0.441	3.86	5.77	-0.05	0.048470	1.314526
Ruminococcaceae	17.338	0.906	10.312	0.300	-4.25	-0.75	-5.85	0.045077	1.346044
Lachnospiraceae	19.520	2.564	10.429	6.571	-2.92	-0.90	-1.57	0.019588	1.708009
Peptostreptococcaceae	0.642	1.306	0.251	0.331	1.02	-1.35	-0.95	0.036126	1.442180
Enterococcaceae	0.240	6.572	6.471	0.202	4.77	4.75	-0.25	0.008922	2.049537
Lactobacillaceae	0.070	0.117	0.042	0.056	0.74	-0.74	-0.33	0.908709	0.041575
Leuconostocaceae	0.005	0.019	0.007	0.000	1.86	0.47	0.00	0.578535	0.237670
Streptococcaceae	0.533	0.922	0.124	0.288	0.79	-2.10	-1.66	0.050969	1.292693
Species level									
Bacteroides massiliensis	1.699	0.001	0.020	0.859	-10.76	-6.38	-0.98	0.130978	0.882801
Bacteroides ovatus	0.465	0.141	0.564	0.605	-1.72	0.28	0.38	0.073134	1.135880
Bacteroides uniformis	2.192	0.003	0.048	0.647	-9.12	-5.49	-1.76	0.041981	1.376947
Roseburia faecis	4.479	0.066	2.005	0.004	-6.07	-1.16	-10.02	0.102169	0.990680
Faecalibacterium prausᵍ	8.852	0.196	5.058	0.020	-5.49	-0.81	-8.75	0.004252	2.371406
*Escherichia;*Other	0.036	6.325	4.008	52.824	7.43	6.77	10.49	0.576626	0.239105
*Klebsiella;*Other	0.134	10.631	1.580	0.380	6.30	3.55	1.50	0.043242	1.364094
Klebsiella;s	0.113	32.130	1.296	0.407	8.15	3.51	1.85	0.646024	0.189751

*Sequencing of each sample was obtained from pooled DNA of two different experiments. The two experiments were performed with two sets of pools of colon microbiotas from two lactose intolerant certificated volunteers; [#] Constructed from Biome files; [§] *Faecalibacterium prausnitzii*

ANOVA for group comparison of BL means and EP values. BL = Baseline; EP = Endpoint; L = milk with lactose; LF = milk lactose-free; BC = Blank control

Metataxonomy data of HCM at the family level were filtered to discuss those families involved in milk fermentation, and the results demonstrated that some taxa were not affected by the fermentations of both the milk samples, while others were modulated on a time and substrate dependency (Table 2). For example, amongst those that did not significantly change it is of interest to mention the *Lactobacillaceae* and the *Streptococcaceae*. Such feature could be attributable to their specialization in metabolization of different dairy sugars.

Among those families that were significantly affected by milk fermentations, *Enterobacteriaceae* were overrepresented at any EP of any sample, with a prominence for L, but not significantly in respect to LF. The culprits of the recorded surges were mostly species of genus *Escherichia* and

Klebsiella, with the exclusion of pathogenic ones that did not match from the sequencing database. In particular, the increment observed of two *Klebsiella* taxa were averagely double in L than in LF. *Enterobacteriaceae* is avid of any dairy carbohydrate (Hervert et al., 2017) and makes no selective differences. Also, *Bacteroidaceae* were significantly modulated by colonic fermentation, as they were underrepresented by any milk substrate fermentation, but that of L accounted for the top reduction of about 5 more times lower in respect to the BC. For example, *Bacteroides uniformis* was reduced 9.12 folds after fermentation of L, almost twice stronger than LF.
In this situation the results are clearer, evidencing that these important butyrate-producer commensals were more underrepresented when exposed to lactose, as a results of the higher innate symbiosis to the lactose intolerant host ecosystem that makes them unable to face the lactose insult and utilize other sugars. Such more negative effect of L in respect to LF fermentation is confirmed at the species level with the higher depletion of renown health-related taxa, as *Faecalibacterium prausnitzii* (6.7 times more) and *Roseburia faecis* (5.5 times more). Similar trends were also seen in vivo models with lactose intolerant microbiota (Ntemiri et al., 2019; Xue et al., 2020). Still among *Clostridiales*, it is to notion the changes observed in the *Peptostreptococcaceae* family, which includes several pathogens (Milani et al., 2016). From our results this family raised just with the fermentation of milk with lactose.

Conclusion

The recipient results have demonstrated that in an *in vitro* colon model of lactose intolerant adults, the fermentation of lactose resulted in an effective insult for the host colonic microbiota.Such effect is documented by the depletion of commensals butyrate producers (*Ruminococcaceae* and *Lachnospiraceae*), and commensal fibrolytic *Bacteroidaceae* and also by the raise in dysbiotic and diarrhea inducers (either at the phylum and family levels, i.e.*Proteobacteria* and *Enterobacteriaceae*, and by the raise of opportunistic *Peptostreptococcaceae.* The impact of the presence of lactose in the colonic microbiota of lactose intolerant adults seems not to affect those bacterial groups more specialized in metabolizing dairy relative sugars, as those are innately adapted to switch their metabolism to different sugars substrates, such as *Lactobacillaceae*, *Streptococcaceae*, and *Bifidobacteriaceae*.
In contrast, lactose intake seems to affect more that part of the microbiota less specialized in the uptake of dairy sugars. In particular, it affects those commensal groups which are intimately adapted to host, i.e the ecosystem of intolerants adults and consequently, by selective pressure havesilenced the expression of enzymes for dairy sugars degradation.
Considering, the changes in the metabolites production during colonic fermentation, we evidenced the negative effect of lactose intake towards the colonic microbiota of lactose intolerant adults, as the reduction in production of Butanoic acid, possibly linked to the depletion of butyrate-producers taxa. This work supports the idea of a microbiota mediation of lactose intolerance and provide a valuable approach to furtherly study disorders mediated by gut microbiota. Notwithstanding, some perspective for future research will be that of investigating the carbohydrate active enzymes expression of the colonic microbiota of lactose intolerant adults, and also to translate these *in vitro* experiments on a healthy human model.

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