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## Session II: Food microbes facing the challenges of healthy human nutrition

### ***Lactobacillus plantarum* exerts in vitro anticancer activities by producing butyric acid: a genome-scale investigation behind this health-promoting metabolic pathway**

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### **Introduction**

Research dealing on butyrogenic bacteria, i.e. capable to produce butyric acid, has gained considerable attention in recent years, since butyric acid exerts an extensive influence on host physiology and gut homeostasis in humans by preventing several intestinal metabolic syndromes and their consequential severe degenerations, first of all the colorectal cancer (CRC). In this frame it is noteworthy that CRC-associated microbiota is modified by probiotic administration, which may represent a relative cheap intervention, capable to enrich the abundance of butyrogenic bacteria in the mucosal layer (Hibberd et al., 2017). This Short Chain Fatty Acid (SCFA) is the end-product of the intestinal microbial fermentation of undigested dietary fibres, which is mainly ascribed to members of *Firmicutes*, such as *Lachnospiraceae*, *Ruminococcaceae* and *Clostridium spp.* Potential butyrogenic capability of Human Intestinal Microbiome (HIM) is currently inferred by amplicon-based sequencing and metagenomics. These approaches target specific key genes of butanoate pathway that encode for the final enzymatic conversion of butyryl-CoA to butyric acid via transferases and butyrate kinase (Vital et al., 2014). As for all *Lactobacillus* sp. that transiently colonize the gut microbiota, *Lactobacillus (L.) plantarum* is considered both an indirect and direct butyrogenic species, which may certainly acts by favouring butyric acid production of other bacteria or directly producing this SCFA in a strain-dependent manner and in response to specific nutrient requirements. In accordance with the fermentation of fibres occurring in the large intestine, production of butyric acid in *L. plantarum* seems triggered by fibres like fructooligosaccharides (FOS) and inulin (Esquivel-Elizondo et al., 2017). Although the *L. plantarum* genome and its metabolic pathways are being reconstructed and studied from many years, so far no specific metabolic route has been ascribed to the production of butyric acid.

Therefore, the present study was aimed to unravel the butyrogenic pathway of *L. plantarum* and its triggering environmental factors, as well as the extend by which this metabolic feature may determine the anti-cancer activity of this species.

### **Material and methods**

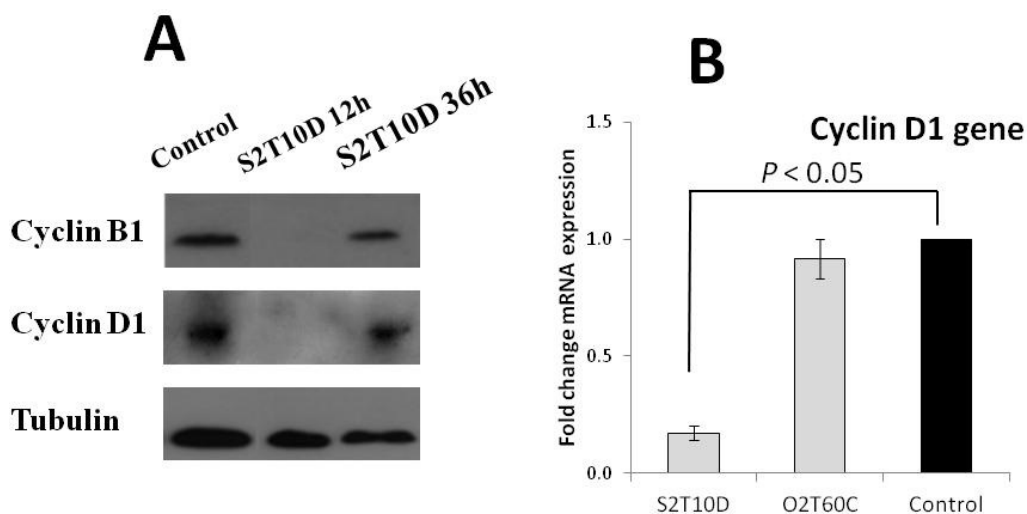
The putative probiotic strains *Lactobacillus plantarum* O2T60C, S11T3E and S2T10D (Botta et al., 2014) were chosen, in relation to their different strains-associated butyrogenic capability in substrates not enriched with fibres (Botta et al., 2015; Pessione et al., 2015).

Their anti-proliferative activity were assessed on HT-29 and Caco-2 cells, by means of SRB assay, MTT assay and cells counting. Subsequently, to the define whether the growth inhibitory activities were related to a modification of aberrant cells cycle or due to the apoptotic cascade activation, we carried out a targeted transcriptomic investigation by using RT-qPCR. The observed transcriptional response of the cells to the bacterial treatments were thus confirmed with flow cytometric analysis and western blot assay. The organic acid profiles of these strains in the cells culture medium, DMEM, were in parallel analyzed by HPLC-UV-RI.

Moreover, the whole genomes of the three strains were sequenced, de novo assembled and annotated to allow a comparative and functional genomics investigation between the butyrogenic and non-butyrogenic strains. The genomics comparison was performed up to the level of single nucleotide polymorphisms (SNPs) analysis and consequent prediction of protein damaged functionality.

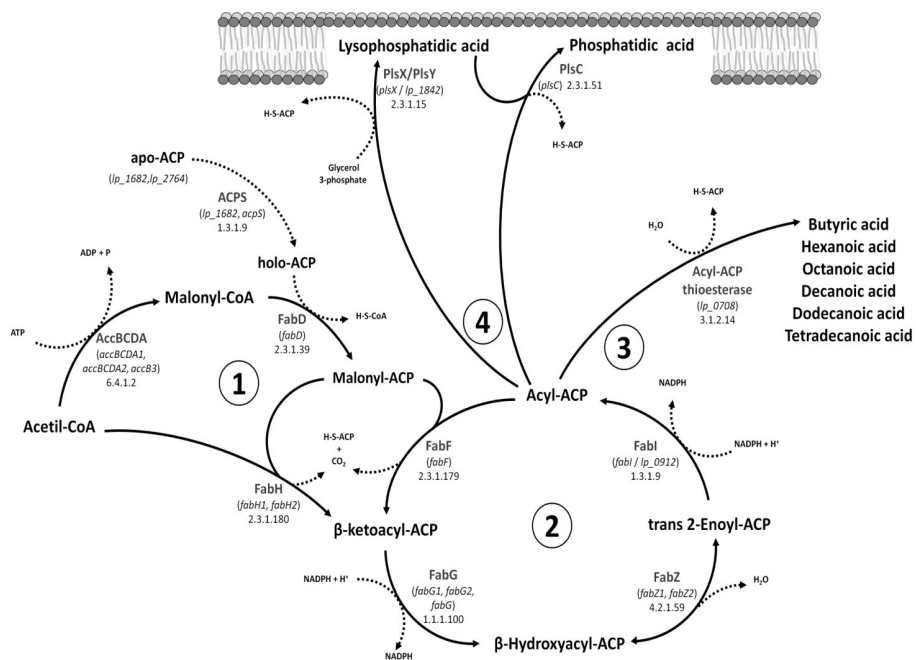
## Results

Overall, the strains significantly inhibited the growth of HT-29 colon cancer cells and their antiproliferative activities were strongly correlated to the type and amounts of extracellular metabolites released in the culture media: a high-glutamine supplemented DMEM. Notably, the supernatants of *L. plantarum* O2T60C poorly inhibited the growth of cancer cells, likely due to its inability to produce butyric acid. On the other hand, the butyrate-containing supernatants of S2T10D induced a significant pH-independent growth inhibition of cancer cells, which was accompanied by a modulation of cyclin D1, and a subsequent cell cycle arrest (Figure 1).



**Figure 1.** Results of co-incubation treatments of HT-29 cells (from human CRC) with CFSn of S2T10D and O2T60C. Cyclin D1 and B1 protein levels(A), cyclin D1 gene expression levels after 12 hours of incubation (B).

Analyzing the genomes of the three strains we attributed, for the first time the production of butyric acid in *L. plantarum* species, to the complementary activities of the type two fatty acid synthase (FASII) and a medium-chain thioesterase (Figure 2). Interestingly, we did not observe differences at genomic, structural and SNPs levels in FASII pathway of butyrate-producing strains compared to strain O2T60C, in which however we predicted several deleterious mutations for genes responsible of glutamine/glutamate metabolism. This *in silico* observations well correlated with the metabolic stress suffered by O2T60 in high-glutamine supplemented DMEM, in which the strain was incapable of producing butyrate. On the other hand the glutamine triggered butyric acid production in S2T1D and S11T3E.



**Figure 2.** Proposed butyrogenic pathway in *L. plantarum* species. The medium -chain thioesterase (TE) acts by truncating the fatty acid synthase of type II (FASII) after the first elongation cycle .

## Discussion

Our results suggest a cell cycle modulation and consequently a growth arrest of CRC cells co-cultured with the supernatants of butyrogenic strains of *L. plantarum*, which act mainly on the expressed level of cyclin D1 and B1. However, the mechanism underlying these anticancer activities seems to be multifaceted and generally correlated to the bacterial growth. Nevertheless, a clear involvement of butyric acid in the anti-proliferation activity of *L. plantarum* towards cancer cells was here proposed for the first time. The comparative and functional genomics assessment of butyrogenic and non-butyrogenic strains of *L. plantarum* allowed us to ascribe this important metabolic activity to the FASII pathway, and identified the glutamine as a powerful triggering nutritional component different from fibres.

Regardless from the limited impact of a butyrogenic activity of *L. plantarum* in DMEM, the outcomes of this study may lead to reconsider the role played by this species and other commensal lactobacilli in the intestinal butyrogenesis and prevention of colorectal cancer.

## Keywords

Anticancer activity, butyric acid, *Lactobacillus plantarum*, comparative genomics, metabolic pathway

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