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*Full Length Research Paper*

# Impact of purified human milk oligosaccharides as a sole carbon source on the growth of lactobacilli in *in vitro* model

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**Recently, there is a growing interest in the use of oligosaccharides as prebiotics in order to modulate the growth of beneficial gut microbiota. It is known that human milk is a rich source of complex oligosaccharides. This paper reports the *in vitro* growth of six strains of lactobacilli in media containing purified human milk oligosaccharides (HMOs) obtained from breast milk. Based on the evaluation of bacterial densities in the growth media, together with the evaluation of pH values and bacterial metabolite detection, we concluded that the lactobacilli tested did not appear to be active HMO consumers. In the case of four strains (*Lbc. fermentum*, *Lbc. animalis* and two strains of *Lbc. delbrueckii* subsp. *bulgaricus*), no increase in bacterial density was detected. Two strains (*Lbc. acidophilus* and *Lbc. casei* subsp. *paracasei*) showed a slight, but insignificant increase in bacterial densities during 24 h of incubation.**

**Key words:** Bifidobacteria, human milk oligosaccharides, lactobacilli, utilization.

## INTRODUCTION

Human milk is a dynamic biological system (Bertino et al., 2009) containing nutrients such as proteins, lactose, fatty acids, and others, as well as biomolecules having prebiotic, immunomodulatory, or antimicrobial effects. From this group, human milk oligosaccharides (HMOs) are thought to have an important role, especially in infant nutrition.

HMOs represent the third most abundant component in human milk (Casado et al., 2009), after lactose and lipids. The content of HMOs is estimated to make between 5 to 23 g/l (Ninonuevo and Lebrilla, 2009) depending on the lactation phase, genetic factors, dietary, geographical factors, and individual determinants (German et al., 2008).

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Maximum concentrations are present in colostrums, while in mature milk, contents of approximately 12 to 14 g/l are detected (Coppa et al., 2006). HMOs are composed by the following monosaccharides: glucose, galactose, sialic acid, fucose and N-acetylglucosamine (Garrido et al., 2012). Many diverse combinations and compositions of these monosaccharides, as well as several combinations of glycosidic bonds, contribute to the complexity of HMO structures (Ninonuevo and Lebrilla, 2009).

Various functions of HMOs are described in literature. They seem to have important functions in the development of the intestinal epithelium of infants (Lara-Villoslada et al., 2006), in establishing a healthy microbiota (Ninonuevo and Lebrilla, 2009), in acting as pathogen receptors (Barile and Rastall, 2013), and in having immunomodulatory properties (Venema, 2012). They are also an important source of monosaccharides, – as they provide glucose as an energy source (Venema, 2012), and sialic acid for neural tissue and brain development. One of the most important functions of HMOs is the prebiotic (bifidogenic) effect. They seem to play a key role in promoting a bifidobacteria-dominant microbiota in newborns (Coppa et al., 2006). Prebiotics influence the host by stimulating the growth and/or activity of beneficial microbiota already established in the colon (Roberfroid, 2007). The potential bifidogenic effect of breast milk was already observed and published by György et al., in 1954 (Ward et al., 2007). Since then, many other works have supported this hypothesis, and further specified that this bifidogenic effect is linked especially to oligosaccharides present in human milk (Han et al., 2012). HMOs have been proved to selectively stimulate the growth of specific bifidobacterial strains, preferentially *Bif. longum* biovar *infantis* and *Bif. bifidum,* which grew successfully on purified HMOs as the sole carbon source (Ward et al., 2006, 2007; LoCascio et al., 2007; Marcobal et al., 2010; Rockova et al., 2011a,b). It is generally accepted that HMOs have prebiotic effects, selectively serving as a source of energy for desired bacteria in the infant intestine (Bode, 2009). However, research on the capability of utilizing HMOs is mainly focused on bifidobacteria – as the predominant bacterial group in the infants' gut. Data on the utilization of HMOs by other intestinal microorganisms, among others also lactobacilli, as beneficial bacteria is scarce. As demonstrated by Marcobal et al. (2010), aside from bifidobacteria, some other intestinal bacteria are able to metabolize HMOs, including *Bacteroides fragilis* and *Bacteroides vulgatus*. These strains were proved to metabolize HMOs with high efficiency in *in vitro* conditions.

From the genus *Lactobacillus*, only strains *Lbc. gasseri* ATCC33323 (Ward et al., 2006) and *Lbc. acidophilus* NCFM (Marcobal et al., 2010) were tested for their ability to grow on HMOs. In the case of *Lbc. gasseri,* no growth was observed, whereas *Lbc. acidophilus* showed weak, but noticeable growth. No more information on the ability of lactobacilli to utilize HMOs is available according to our knowledge. The aim of this study was to investigate the ability of several strains of lactobacilli to ferment HMOs as a sole carbon source in *in vitro* conditions, thus furthering our knowledge regarding the selectivity of HMOs.

## MATERIALS AND METHODS

### Bacterial strains

The list of strains (six strains of lactobacilli and one strain of bifidobacteria) used in this work is shown in Tables 1 and 2. The strains were procured from the Culture Collection of Dairy Microorganisms Laktoflora® - CCDM (Prague, Czech Republic), from the Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources of the Czech University of Life Sciences in Prague. Human isolates of lactobacilli were obtained from biopsy samples (Dairy Research Institute Tábor, Czech Republic).

### Isolation and purification of HMOs

Human milk samples obtained from three different donors, kindly provided by the Gynecology and Obstetrics Clinic of Charles University and the General Faculty Hospital in Prague, were used for the isolation and purification of HMOs. Oligosaccharides were extracted according to the methodology described by Gnoth et al. (2000), with a few modifications. In the first step, milk (100 ml) was centrifuged at 1800 *g* for 30 min at 4°C, thus, lipids, proteins and cells were partially removed. Subsequently, proteins were precipitated by the addition of ethanol (2:1, v/v). The solution was stored at 4°C for 24 h. After centrifugation (under above mentioned conditions), the solvent was removed by rotatory evaporation, and the remainder of the solution was dissolved in deionized water. The whole process of precipitation was repeated twice. Gel filtration chromatography on a column filled with Toyopearl HW40F in 1% acetic acid (flow rate 0.1 ml/min) was used. The eluate was collected in 2.5 ml fractions and screened for the presence of oligosaccharides by thin-layer chromatogramy using isopropanol : water : 25% ammonia solution (5:1:2, by volume) as a mobile phase, and was then visualized by spraying with 10% sulphuric acid in ethanol and heating. Carbohydrate containing fractions (a total volume of 50 ml) were dispensed into vials and cooled at a temperature of 4-8°C for 30 min., and frozen at – 70°C for 90 min. Samples were subsequently lyophilized using Cryodos device (Telstar, Spain). The yield from 100 ml of milk made 0.5 g of purified oligosaccharides.

### Bacterial growth on HMOs

Basal medium (tryptone, 10 g; peptone, 10 g; yeast extract, 5 g; Tween 80® 1 ml, distilled water 1 L) was autoclaved (121°C, 15 min). Purified oligosaccharides (1 % w/w) were added as a sole carbon source to the cooled medium after sterile filtration (Puradisc FP 30 filter 0.2 μm, Whatman, Germany). As a negative control, a medium devoid of carbohydrate was used. As a positive control, Wilkins Chalgren broth (Oxoid, Basingstoke, UK) was used. Overnight bacterial cultures were centrifuged (5000 *g*, 7 min) and re-suspended in saline. Bacterial suspensions were inoculated into

**Table 1.** Utilization of human milk oligosaccharides.

| Strain | Density of lactobacilli (change in $A_{540}$) | | |
|---|---|---|---|
| | HMO | BM | WCH |
| *Lbc. fermentum* RL 25 | 0.13 ± 0.06$^{ab}$ | 0.17 ± 0.12$^{b}$ | 5.20 ± 0.20$^{c}$ |
| *Lbc. delbrueckii* subsp. *bulgaricus* CCDM 66 | 0.07 ± 0.06$^{a}$ | 0.03 ± 0.06$^{a}$ | 5.03 ± 0.21$^{bc}$ |
| *Lbc. acidophilus* CCDM 151 | 0.53 ± 0.06$^{c}$ | 0.07 ± 0.06$^{ab}$ | 4.87 ± 0.06$^{b}$ |
| *Lbc. delbrueckii* subsp. *bulgaricus* CCDM 767 | 0.03 ± 0.06$^{a}$ | 0.03 ± 0.06$^{a}$ | 4.77 ± 0.21$^{b}$ |
| *Lbc. casei* subsp. *paracasei* PE1TB-P | 0.43 ± 0.06$^{bc}$ | 0.17 ± 0.06$^{b}$ | 4.97 ± 0.15$^{bc}$ |
| *Lbc. animalis* CCDM 382 | 0.17 ± 0.06$^{ab}$ | 0.07 ± 0.06$^{ab}$ | 5.17 ± 0.15$^{c}$ |
| Average | 0.23 ± 0.20$^{\beta}$ | 0.09 ± 0.08$^{\alpha}$ | 5.00 ± 0.21$^{\gamma}$ |
| *Bif. bifidum* JKM | 2.01 ± 0.47$^{d}$ | 0.07 ± 0.02$^{ab}$ | 3.01 ± 0.11$^{a}$ |

Data are expressed as increase in turbidity of bacterial suspension estimated from increase in A540 during 24 h of incubation; values are means from triplicate determination ± standard deviation (SD). HMO, medium containing purified human milk oligosaccharides as a carbon source; WCH, Wilkins Chalgren broth (control medium); BM, basal medium without carbohydrate source (negative control). a-d data in columns with different superscripts differ ($P < 0.05$). αβγ data in lines with different superscripts differ ($P < 0.05$).

**Table 2.** pH values of media.

| Strain | Origin | Final pH values after 24 h of incubation | |
|---|---|---|---|
| | | HMO | WCH |
| *Lbc. fermentum* RL 25 | human faeces | 6.20 ± 0.03$^{b}$ | 4.86 ± 0.04$^{f}$ |
| *Lbc. delbrueckii* subsp. *bulgaricus* CCDM 66 | yogurt, Turkey | 6.50 ± 0.04$^{cd}$ | 4.56 ± 0.04$^{b}$ |
| *Lbc. acidophilus* CCDM 151 | pill Biolacta | 6.14 ± 0.02$^{b}$ | 4.66 ± 0.05$^{d}$ |
| *Lbc. delbrueckii* subsp. *bulgaricus* CCDM 767 | yogurt, Switzerland | 6.55 ± 0.03$^{d}$ | 4.86 ± 0.04$^{f}$ |
| *Lbc. casei* subsp. *paracasei* PE1TB-P | biopsy sample (colon) | 6.20 ± 0.05$^{b}$ | 4.54 ± 0.05$^{a}$ |
| *Lbc. animalis* CCDM 382 | raw goat milk | 6.45 ± 0.05$^{c}$ | 4.68 ± 0.05$^{e}$ |
| Average | | 6.34 ± 0.17$^{\alpha}$ | 4.69 ± 0.14$^{\beta}$ |
| *Bif. bifidum* JKM | infant faeces | 5.00 ± 0.20$^{a}$ | 4.65 ± 0.15$^{c}$ |

Values are means ± standard deviation (SD) of three measurements. $^{a\text{-}f}$ data in columns with different superscripts differ ($P < 0.05$). $^{\alpha\beta}$ data in lines with different superscripts differ ($P < 0.05$). HMO – medium containing purified human milk oligosaccharides as a carbon source. WCH, Wilkins Chalgren broth (control medium). Initial pH values of HMO and WCH media were 6.60 and 6.40, respectively.

a medium containing HMOs and then incubated at 37°C for 24 h under anaerobic conditions. All strains were grown in triplicate. The growth of lactobacilli was evaluated as the change in absorbance $A_{540}$ during 24 h of incubation by measuring transmitted light using densitometer DEN-1 (Dynex, Czech Republic). Results were expressed as increase in turbidity of the bacterial suspension estimated from increase in $A_{540}$. For the determination of pH values, pH meter HACH sension 1 (HACH, USA) was used. The results were evaluated using MS Excel 2007 (Microsoft, Redmond, USA).

#### Determination of bacterial metabolites

To determine organic acids concentration, the isotachophoretic (ITP) method was used. The samples after fermentation by lactobacilli were subjected to isotachophoretic separations using IONOSEP 2003 device (Recman, Czech Republic). The change in the content of lactic acid as the major metabolite of lactobacilli as well as the content of acetic, butyric, propionic, formic and succinic acids was monitored. Prior to analysis, the samples were diluted with 150 volumes of deionized water, and then purified using the Puradisc FP 30 filter with a pore size of 0.2 µm (Whatman, Germany). Solution containing 10 mM HCl, 22 mM ε-aminocaproic acid and 0.1 % 2-hydroxy-ethylcellulose (pH 4.5) as leading electrolyte (LE) was used. As trailing electrolyte (TE), 5 mM caproic acid was used. All chemicals were obtained from Sigma-Aldrich (Czech Republic). The values of the initial and final stream used were 80 and 30 µA, respectively.

#### Statistical analyses

For evaluation of the results Statgraphics® Centurion XV (StatPoint, Inc., Warrenton, USA), the multiple range comparison - LSD test was used. A significant difference was statistically considered at the level of $P < 0.05$.

## RESULTS AND DISCUSSION

In this work, 6 strains of lactobacilli of different origin were tested for their ability to ferment HMOs as a sole

carbohydrate source. The growth of strains tested is summarised in Table 1. In the case of the four strains (*Lbc. fermentum* RL25, *Lbc. animalis* CCDM 382 and two strains of *Lbc. delbrueckii* subsp. *bulgaricus* CCDM 66 and CCDM 767), no increase in bacterial density in the medium with HMOs was observed. The change in the absorbance $A_{540}$ after 24 h of incubation in these groups of strains ranged from 0.03 to 0.17. In the rest of the strains tested (*Lbc. acidophilus* CCDM 151 and *Lbc. casei* subsp. *paracasei* PE1TB-P), a slight increase in bacterial densities in HMO-containing medium was observed (0.53 for *Lbc. acidophilus*, 0.43 for *Lbc. casei* subsp. *paracasei*). As a positive control, Wilkins Chalgren (WCH) broth was used. In this medium, high cell densities (from 4.77 to 5.20) in all strains were obtained (Table 1). The strain *Bif. bifidum* JKM was used as a positive control, too. This strain is able to effectively utilize HMOs, as demonstrated previously (Rockova et al., 2011a). As a negative control, a basal medium without any added sugar was used. A marginal increase in absorbance $A_{540,}$ even in the absence of sugar, was seen (Table 1). Increased cell numbers for bacterial species like *Lactobacillus, Enterococcus, Enterobacteriaceae* or *Staphylococcus* in media without carbohydrate supplementation were also observed by other authors (Marcobal et al., 2010; Satoh et al., 2013).

The strain PE1TB-P began to grow in WCH broth after the first hour of incubation (Figure 1), while growth in the HMO-containing medium was noticeable after three hours. Instead of exponential growth, a slight steady growth during 24 h of incubation was observed. A very similar trend was noticed for the strain *Lbc. acidophlilus* (data not shown).

To precisely evaluate the fermentation ability, besides measuring the bacterial density, it is important to analyse the changes in pH of growth media, and possibly to analyse metabolite concentration produced by bacteria. Final pH values (Table 2) are consistent with the change in $A_{540}$ measured after 24 h of incubation. The pH of the medium containing purified HMOs decreased from the initial value of 6.60 to 6.34 on average, while in the control medium (WCH), the pH decrease was much more apparent (from 6.40 to 4.69 on average). Anaerobic intestinal microbiota convert carbohydrates to lactic acid and short-chain fatty acids (Loo et al., 1999) such as acetic, propionic and butyric acids. Lactic acid has a role in maintaining lower intestinal pH (Satoh et al., 2013), while butyric acid, sometimes produced by heterofermentative lactic acid bacteria, provides nutrition of the colonic epithelium and has an important role in gut maintenance (Venema, 2012). The results of bacterial metabolite analysis are presented in Figures 2 and 3. The medium with HMOs produced significantly lower concentrations of lactic acid compared to the control medium (WCH broth) after 24 h of fermentation. The production of lactic acid in WCH broth rose to 225 mg/100 ml (in the strain PE1TB-P), while the maximum concentration of lactic acid detected in the medium with HMOs made no more than 40 mg/100 ml (in the strain CCDM 151). To a somewhat lower extent also in the strain PE1TB-P a slight increase in lactic and acetic acids was visible, which indicates some bacterial growth. Concentrations of succinic and formic acids rose marginally (up to 16 and 11 mg/100 ml, respectively), and in the case of propionic and butyric acids, non-detectable concentrations, even lower than 2 mg/100 ml (data not shown), were obtained.

The strain *Bif. bifidum* JKM, used as a positive control, showed very good growth in the medium with HMOs compared to the growth of lactobacilli. The increase in the absorbance $A_{540}$ made 2.01 (Table 1). The growth was accompanied by a decrease in pH values (Table 2) and by an increase of acids produced (Figure 2).

Direct fermentation of HMOs by intestinal microbiota has not yet been well described and there is a lack of information regarding their utilization by specific bacterial species (lactobacilli). The majority of information, that exists on HMO fermentation refers to bifidobacteria as the predominant bacterial group in a healthy infants's gut. Many *in vitro* studies were conducted on the capability of bifidobacteria to ferment HMOs with positive results (Ward et al., 2006, 2007; LoCascio et al., 2007; Marcobal et al., 2010; Satoh et al., 2013), but growth in the presence of HMOs is not a property of all representatives of the genus *Bifidobacterium*. Preferential growth of *Bif. longum* subsp. *infantis,* a species often occurring in infants, was noticed in the aforementioned studies. This strain preferentially utilized oligosaccharides with a degree of polymerization (DP) $\leq$ 7. These oligosaccharides form a significant part of breastmilk (LoCascio et al., 2007). In the study conducted by Rockova et al. (2011a), bifidobacterial strains of human origin (*Bif. bifidum* and *Bif. longum*) were proved to utilize HMOs with high efficiency in comparison with bifidobacteria of animal origin (*Bif. animalis).* Utilization capability is closely related to the enzymatic equipment that specific bacteria possess. Enzyme lacto-N-biose I phosphorylase was recently proved to be responsible for the cleavage of lacto-N-biose I, which is an important component of HMOs (Satoh et al., 2013). The presence of this enzyme was detected in species *Bifidobacterium bifidum* and *Bifidobacterium longum* occuring in infants' gut (Wada et al., 2008). Conversely, in other bacterial groups like lactobacilli, clostridia or bacteroides, this enzyme was not observed (Wada et al., 2008). The strain *Bif. longum* subsp. *infantis* also possesses other enzymes involved in the cleavage of HMOs, such as fucosidase or sialidase (LoCacio et al., 2007). Additionally, between certain bifidobacterial strains, commensal activities were described, where strains able to cleave long-chain HMOs (*Bif. bifidum*) can provide monosaccharides for other strains

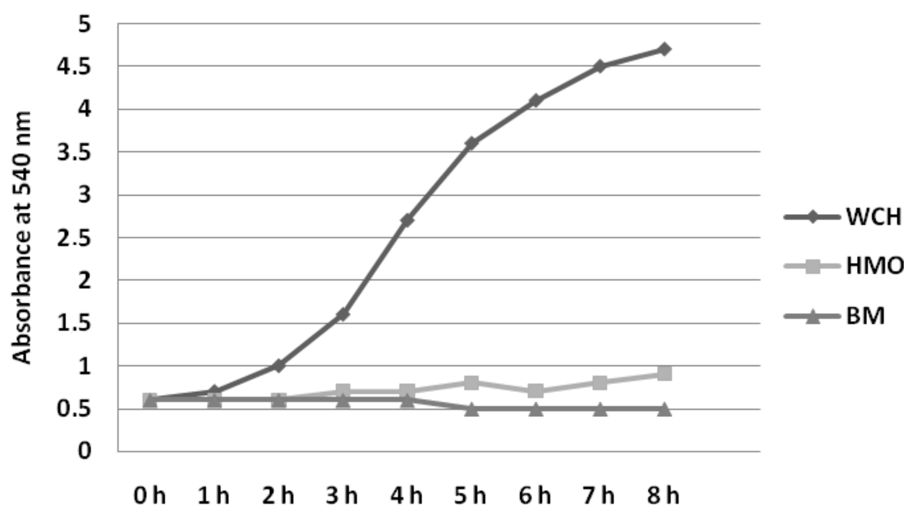


**Figure 1.** Growth of *Lbc. casei* subsp. *paracasei* PE1TB-P in the medium containing HMOs as a sole carbon source. WCH, Wilkins Chalgren medium as a positive control; BM, basal medium without any carbohydrate as a negative control.

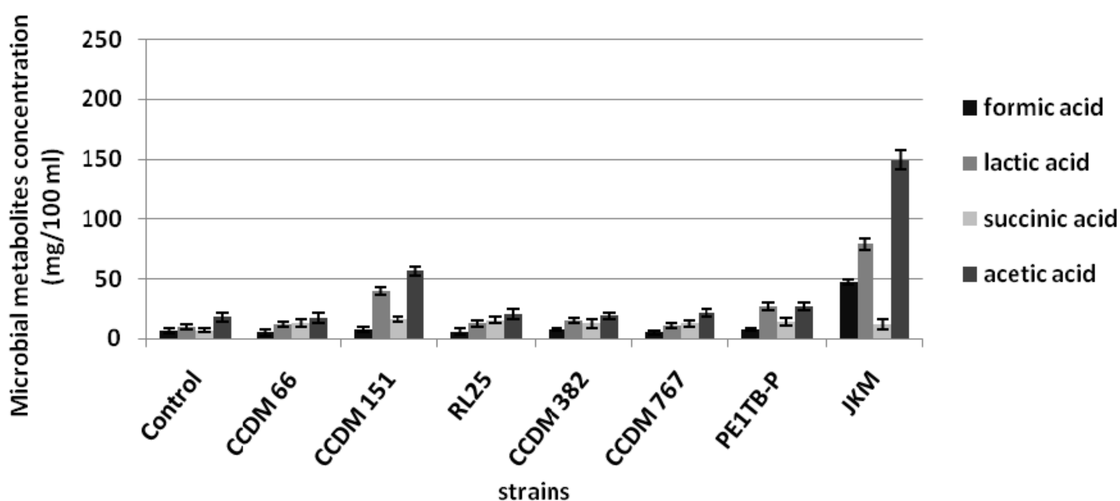


**Figure 2.** Concentrations of microbial metabolites in the medium containing purified human milk oligosaccharides after 24-h fermentation by lactobacilli. Concentrations of butyric acid and propionic acid made less than 2 mg/100 mL of the media (data not shown). Bars present mean ± SD.

(*Bif. breve*, Ward et al., 2007). Marcobal et al. (2010) demonstrated that HMO fermentation is not an exclusive property of specific strains of bifidobacteria. In the study conducted by this group, apart from *Bif. longum* subsp. *infantis*, for the first time, *Bacteroides fragilis* and *Bacteroides vulgatus* were proved to be able to metabolize HMOs with high efficiency. Either weak or no fermentation was exhibited by genera *Clostridium, Eubacterium, Enterococcus, Streptococcus*, *Veillonella* and *E. coli* strains. From the group of lactobacilli, a strain *Lbc. acidophilus* NCFM was tested which showed some growth ability on this substrate (Marcobal et al., 2010). In another *in vitro* study (Ward et al., 2006), a strain *Lbc. gasseri* ATCC33323 was tested in which the ability to ferment HMOs was not proved.

The major part of HMOs reach the colon in unhydrolyzed form, where they may be utilized by intestinal microbiota into short chain fatty acids (Lasrado and Gudipati, 2013) and thus serve as nutrients – prebiotics (Loo et al., 1999; Ninonuevo and Lebrilla, 2009). A prebiotic effect is proven when the growth of beneficial bacteria is stimulated, while potentially harmful bacteria

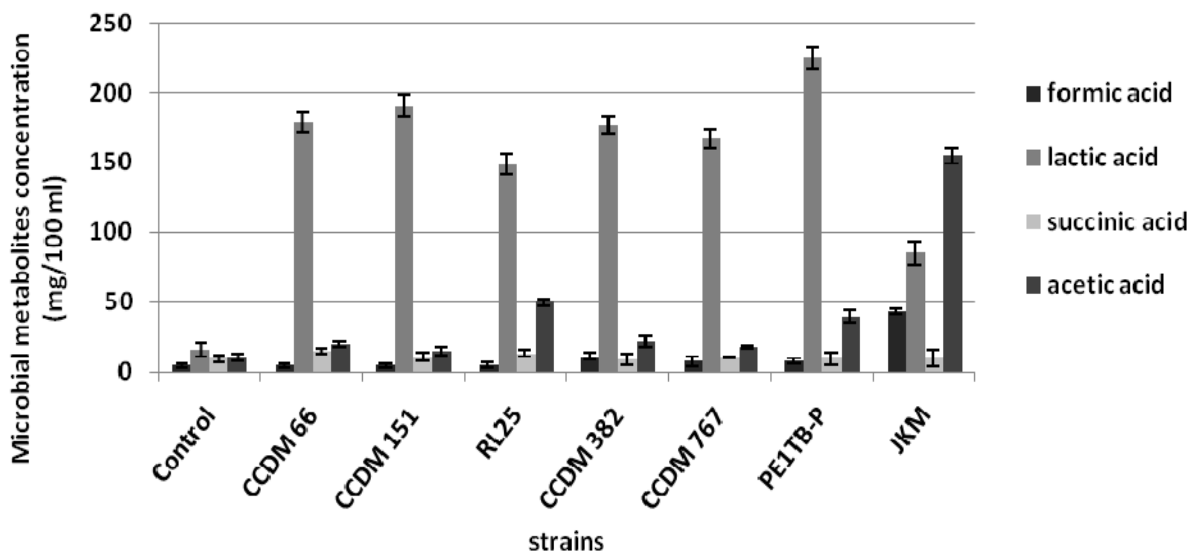


**Figure 3.** Concentrations of microbial metabolites in the medium containing Wilkins Chalgren broth after 24-h fermentation by lactobacilli. Concentrations of butyric acid and propionic acid made less than 2 mg/100 mL of the media (data not shown). Bars present mean ± SD

are inhibited (Boehm et al., 2004). In this study we conducted an *in vitro* testing on direct fermentation of purified HMOs by lactobacilli. The results of this work support the hypothesis that utilisation of HMOs may be species- and strain specific. Based on the evaluation of the results obtained by absorbance $A_{540,}$ measured together with bacterial metabolite detection and the evaluation of pH values, we concluded that the lactobacilli tested did not appear to be active HMO consumers. This fact supports the hypothesis that HMOs may selectively enhance the growth of specific bacterial groups (particularly bifidobacteria) present in the colon of newborns.

## Conflict of Interests

The authors did not declare any conflict of interests.

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