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Characterisation of a *Bifidobacterium* sp. strain isolated from human faeces and its expression of the *ack* and *ldh* genes

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Faecal samples of two Mexican breast-fed infants were used to isolate a *Bifidobacterium* strain in a selective medium. This strain was characterised biochemically for carbohydrate fermentation patterns and the fructose-6-phosphate phosphoketolase assay; genetically it was identified by PCR using genus-specific primers. The isolated strain was named *Bifidobacterium* JCLA3 and was grown on four different carbon sources; the respective growth kinetics of glucose, lactose, inulin and sucrose were measured. The highest cellular yield was presented in the culture media containing sucrose, followed by inulin, while on glucose and lactose, the cellular yield was lowest; these results correlate with the characteristic that bifidobacteria prefer complex carbon sources. We also determined the expression of the *ack* and *ldh* genes. The *ldh* gene presented high levels of expression regarding the *ack* gene; nevertheless on sucrose, the expression was lower regarding the other three substrates. The highest expression level of the *ack* gene was from the culture media with glucose, followed by inulin, sucrose and lactose.

Key words: *Bifidobacterium*, characterisation, gene expression.

INTRODUCTION

Bifidobacteria represent a significant proportion of the infant and adult gut microbiota. The beneficial effects of bifidobacteria on human health have been demonstrated in immunopotentiation, nutrition, prevention of intestinal infections, reduction of intestinal putrefaction as well as being used in functional foods and pharmaceutical products (Gibson and Roberfroid, 1995; Eckburg et al., 2005).

The isolation of bacteria from gut microbiota is important to characterise and analyse differences among strains. Selective and differential media have been described for the isolation of *Bifidobacterium* spp., nevertheless their identification is not clear because it is based on phenotypic characteristics that do not always provide reliable results. It is also known that bifidobacteria can change their morphology depending on the culture medium, growth and culture conditions (Bonaparte et al., 2001; Klein et al., 1998). Colonies have been identified as members of the genus *Bifidobacterium* by the detection of fructose-6-phosphate phosphoketolase (F6PPK) (Grill et al., 1995). However, the test is time consuming and laborious (Scardovi, 1986; Biavati et al., 1992; Tannock, 1999).

The use of 16S rRNA gene sequences to study bacterial phylogeny, taxonomy and genus and species identification has been the most common (Woese, 2000). 16S rRNA-targeted hybridization probes or PCR primers enable rapid and specific detection of a wide range of bacterial species, and have become key procedures in the

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detection of microorganisms (Kaufmann et al., 1997).

Like most intestinal bacteria, bifidobacteria are saccharolytic, they obtain carbon and energy through fermentation of host and dietary carbohydrates and show remarkable adaptations to use and metabolize complex oligosaccharides as carbon and energy sources (Lee and O'Sullivan, 2010).

Bifidobacteria are unable to carry out the usual glycollysis pathway or the hexose monophosphate shunt pathway; they possess only one metabolic pathway for the fermentation of hexoses- the bifidus shunt (De Vries et al., 1967). This pathway is dependent on the presence of fructose-6-phosphate phosphoketolase (F6PPK) (Fandi et al., 2001). Bifidobacteria catabolize a variety of mono- and oligosaccharides (Crociani et al., 1994) released by glycosyl hydrolases, with the formation of acetate, lactate, ethanol and even little amounts of succinate, as end products (Schell et al., 2002).

The main impact of these metabolites in human health is in the colon, decreasing the pH, which helps protect the intestine against pathogen colonization, and colon cancer by reducing the bioavailability of the toxic amines. The presence of acetate also helps the absorption of calcium and magnesium, improving blood flow in the colon and in the liver (Fukuda et al., 2012). Very little is known about the analysis of the expression of genes encoding the enzymes responsible for the production of the main metabolites of bifidobacteria.

The purpose of this study was to isolate a strain of the *Bifidobacterium* genus from human faeces and then characterise it biochemically and genetically, as well as analyse the levels of expression of the *ack* and *ldh* genes, coding for the acetate kinase (EC 2.7.2.1) and lactate dehydrogenase (EC 1.1.1.27) enzymes, respectively. The strain was tested in four different carbon sources.

MATERIALS AND METHODS

Isolation and bacterial identification

Faecal samples were obtained from two Mexican breast-fed infants, and explicit informed oral consent was obtained from their parents. Samples were homogenized in sterilized phosphate-buffered saline (PBS), and 1-mL aliquots were used to inoculate trypticase-phytone-yeast extract broth (TPY) supplemented with 1% Raftilose (Megafarma) (w/v). Samples were incubated at 37°C for 72 h under anaerobic conditions in an anaerobic system (Forma Scientific). Decimal dilutions were made in PBS and aliquots were plated on TPY agar supplemented with Raftilose. Colonies were identified as members of the genus *Bifidobacterium* by Gram staining; cellular morphology, carbohydrate fermentation patterns (API 50 CHL system, BioMerieux) and a fructuose-6-phosphate phosphoketolase (F6PPK) assay (Grill et al., 1995). The identity of a presumptive *Bifidobacterium* isolate was confirmed by genus-specific PCR (Kaufmann et al., 1997). The PCR product was subjected to agarose gel electrophoresis (1%) followed by GelRed stain (Biotium). The fragment was purified with the QIAquick Gel Extraction Kit (Qiagen) and sequenced.

Growth on different carbon sources

An inoculum was prepared in vials with 40 mL of trypticase-phytone-yeast extract broth (TPY) for each of the carbon sources: glucose, lactose, inulin and sucrose. The cultures were incubated in anaerobic conditions at 37°C for 8 h at 200 rpm in an Orbital Incubator Chamber (Gallenkamp). These cultures were used to inoculate fresh medium with their respective carbon sources at an initial optical density of 0.05. They were incubated under the same previous conditions, and samples were collected every 1.5 for 6 h (exponential growth rate). All the cells were harvested by centrifugation at 3,000 xg, washed twice in 0.1 N Tris-HCl pH 7.0 and stored in aliquots at -80°C for further analysis. All the fermentations were carried out in triplicate.

Survival and growth of *Bifidobacterium* JCLA3 at different pH conditions

To investigate the acid resistance of *Bifidobacterium* sp. JCLA3, previous TPY/glucose culture media with cells at pH 2, 3 and 4 were stored for 6 months at -20°C. Defrosted culture media were resuspended and centrifuged at 3,000 xg at 4°C for 5 min and washed twice in saline solution. Two milliliters of each cellular suspension was inoculated into vials with 40 mL of TPY/glucose medium at pH 2.0, 3.0 and 4.0, respectively. Cultures were incubated at 37°C and 200 rpm in anaerobic conditions. Samples were taken every 2 for 8 h of fermentation to determine the pH, optical density (OD) and cellular viability.

DNA and RNA isolation

Purified DNA of the isolated bacteria was obtained from a TPY/glucose culture, using the Wizard Genomic DNA Purification Kit (Promega). Samples of the strain growing on glucose, lactose, inulin and sucrose were collected in the exponential phase; RNA was isolated according to the protocol SV Total RNA Isolation System (Promega). The concentration of isolated DNA and RNA was determined using a NanoDrop spectrophotometer (Thermo Scientific, USA) and its quality was analysed by agarose gel electrophoresis (1%) with GelRed (Biotium).

Sequence analysis

The sequence of the purified fragment was determined by the dideoxy chain termination method, using the protocol provided in the Sequenase DNA sequencing kit (US Biochemical Corp.). To determine the close relatives of the partial 16S rDNA sequences, a search in the GenBank DNA database was conducted using the BLAST algorithm.

Expression of the *ack* and *ldh* genes by RT-PCR

For expression experiments, primers to amplify a 245-bp fragment of the *ack* gene were designed using the sequence reported for *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (GenBankNC_017219), forward: 5'-CTCGGCTTCTTCGAGGAGTA-3', reverse: 5'-AAGGAGGAGTCGAACACGAA-3'. For the analysis of the *ldh* gene, two primers were designed to amplify a 208-bp fragment using the sequence of *Bifidobacterium infantis* (GenBank: FJ455841.1) forward: 5'-CACGGCTCCAGCTTCTACTC-3', reverse: 5'-TGAGCATGTAGATGGCGTTC-3'. As a housekeeping gene, a fragment of the constitutive gene xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (*xfp*) was used. Primers were designed from *B. longum* sequence (GenBank: AY377410), forward: 5'-CGGCCACGGCTGGGGCC-3', reverse: 5'-TCCTGACGCCAGACGTGGG-3'.

The expression levels of *ldh, ack* and *xfp* genes were analysed through the One Step Reverse Transcription kit (Qiagen) following the manufacturer's instructions. An amount of 0.3 µg of total RNA

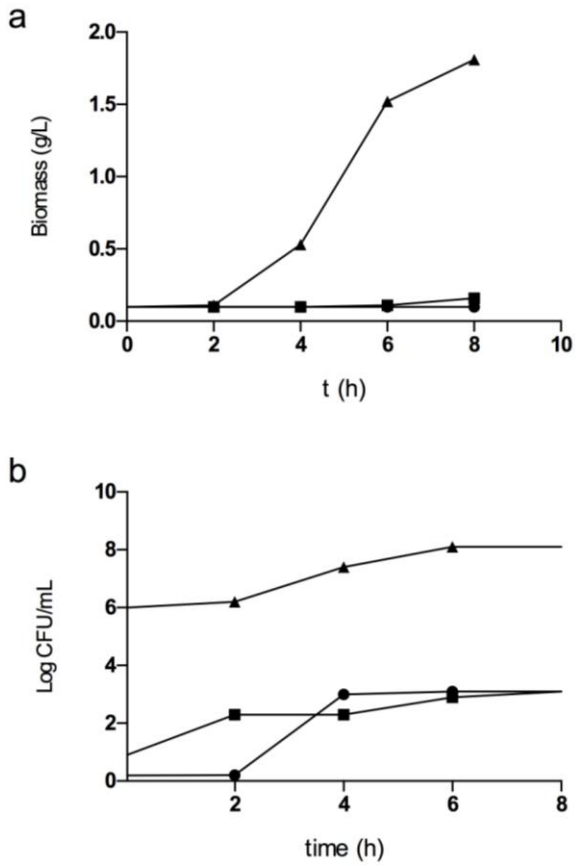

Figure 1. Kinetics of growth (1a) and viability (1b) of *Bifidobacterium* sp. JLCA3 stored for 6 months at -20°C, pH 2.0 (σ), pH 3.0 (ν) and pH 4.0 (●).

was used for each reaction. Reaction conditions were: with reverse transcriptase Superscript II at 50°C for 30 min, followed by specific amplification of the genes (5 min at 94°C, followed by 30 cycles at 94°C for 60 s, 54°C for 60 s and 72°C for 1 min). After amplification, RT-PCR products were analysed in 1.5% agarose gels stained with GelRed (Biotium) for luminescence in a UV transilluminator (Syngene).

Amplicons (cDNA bands) were analysed by densitometry with Kodak Digital Science 1D 3.6 software. Numeric values for the cDNA band intensities of the *Idh* and *ack* genes were normalized with the values of the *xfp* gene, as these express themselves at a relatively constant level in cells and are generally used in semi-quantitative systems of RT-PCR for analysing the relative efficiency of each individual PCR.

RESULTS

The isolated strain from breast-fed infant faeces was *Bifidobacterium* sp. strain JCLA3. This strain was F6PPK-positive and identified as *Bifidobacterium* which was confirmed by carbohydrate fermentation patterns using the API 50 CHL system and by PCR tools for molecular identification at the genus level. Nucleotide sequences were analysed using the BLAST tool. Approximately 30 sequences of different species of *Bifidobacterium* presented high similarity, as well as two sequences of *Gardnerella vaginalis,* which has a close phylogenetic relationship with the genus *Bifidobacterium* (Gavini et al., 1996). Based on the distinct phenotypic characteristics, Gram stain, biochemical and molecular genetics evidence, we confirm that the isolated strain belongs to the genus *Bifidobacterium.*

Growth kinetics

Comparative batch cultures of *Bifidobacterium* sp. JCLA3 on glucose, lactose, inulin or sucrose as sole carbon source were carried out. The strain was incapable of growing in TPY without the addition of carbohydrates. Increment of biomass was followed for 8 h identifying the exponential growth phase. The highest dry cell weight (DCW) production was found on sucrose (1.2 g/L) followed by inulin (0.32 g/L), while on glucose and lactose, the cellular production was 0.21 and 0.18 g/L, respectively. The cellular production was independent of the preference of each strain by the substrate; maximum specific rates for every culture were 0.492, 0.433, 0.275 and 0.274 h^{-1} on glucose, sucrose, lactose and inulin, respectively.

Survival and growth of *Bifidobacterium* sp. JCLA3 at different pH conditions

Bifidobacterium sp. JCLA3, stored at pH 2 at -20°C, maintained a viability of 10^6 to 10^8 CFU/mL after 6 months, whereas the viability of cells stored at pH 3.0 and 4.0 decreased 3 to 4 orders of magnitude. Each strain obtained at a different pH was inoculated on TPY/glucose medium; after 6 h of incubation, the cells at pH 2.0 increased to a cellular density of 1.8 g/L, whereas in the cultures of cells at pH 3.0 and 4.0, the growth was not detected (Figure 1a). The cell viability in the culture at pH 2.0 increased by 2 orders of magnitude after 6 h, unlike cells that were grown at pH 3.0 and 4.0 (Figure 1b), which did not show significant growth.

Expression of the genes *ack* and *Idh*

The expression of genes coding for acetate kinase (*ack*) and lactate dehydrogenase (*Idh*) enzymes were analysed by RT-PCR (Figure 2A and 2B, respectively). The RNA was obtained from the isolated bacteria *Bifidobacterium* sp. JCLA3 during its exponential growth phase on each of the four carbon sources. Comparing the expression levels of the *ack* gene, the highest expression appeared in glucose (42%), followed by inulin (33%), sucrose (29%) and lactose (13%). For the *Idh* gene, the expression levels were high in all cases, similar to the control gene: glucose (97%), followed by lactose and inulin (96%), and sucrose (71%) (Figure 2C). These numeric values for band intensity of cDNA were corrected with the values of the housekeeping *xfp* gene in order to analyse the relative expression of each gene (Figure 2C). With this internal control, it can be demonstrated that the same amount of RNA was used in every experiment, and differences in the expres-

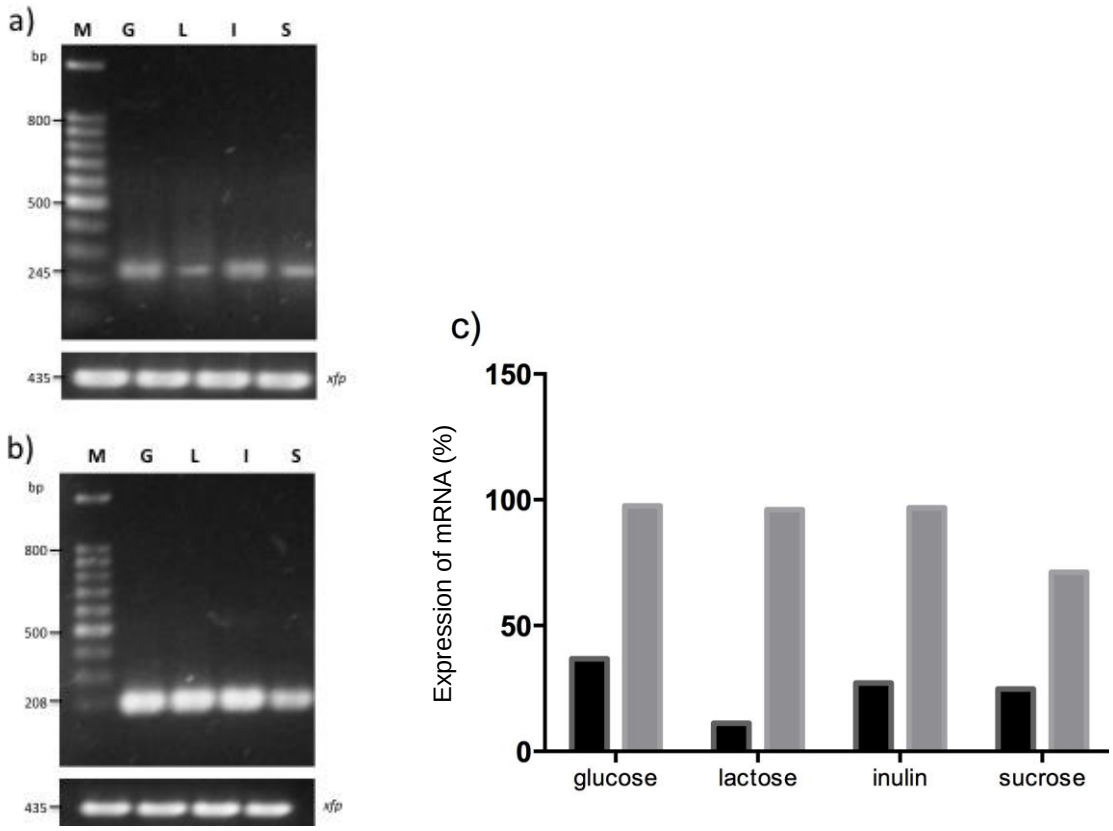

Figure 2. Expression of the *ack* (■) and *ldh* (■) genes by RT-PCR during exponential growth of the *Bifidobacterium* strain isolated from human faeces on G: glucose, L: lactose, I: inulin, and S: sucrose as carbon sources. Amplicons from 300 ng of RNA of the 245-bp *ack* (a) and 208-bp *ldh* fragments (b). Agarose gels of 1.5% stained with GelRed®. M: 100-bp DNA ladder. (c) Graphics representing percentage expression analyses by densitometry; the *xfp* housekeeping gene was used to normalize the data.

sion levels of *ack* were caused by the source of carbon used by *Bifidobacterium* sp. JCLA3.

DISCUSSION

Strain isolation of the genus *Bifidobacterium* from biological samples of the gastrointestinal tract, faeces and dairy products; have been carried out successfully in selective media developed over several decades. However, in order to differentiate them from other morphologically similar bacteria, such as *Lactobacillus, Actinomyces, Propionibacterium and Eubacterium,* biochemical identification by carbohydrate fermentation profiles and the presence of the fructose-6-phosphate phosphoketolase enzyme is important. Nowadays, the use of molecular tools for the detection, differentiation and identification of bifidobacteria has allowed surpassing the limitations that isolation from culture media involved. The design of genus-specific primers based upon the sequence of the 16S rRNA gene is the main analysis conducted in order to identify this microorganism (Kaufmann et al., 1997).

BLAST analysis of the 1.35-kbp fragment sequence amplified by PCR using these primers has allowed us to confirm that the isolated microorganism belongs to the genus *Bifidobacterium*. Nevertheless, the two sequences of *G. vaginalis,* which also showed high similarity cannot be considered due to the fact that this bacteria belongs to the *Bifidobacteriaceae* family, but they are Gram-negative bacilli.

The highest cell production of *Bifidobacterium* sp. JCLA3 was obtained in the sucrose and inulin media, while in glucose and lactose the cell production was low. Previous reports with mono- and oligosaccharides using different bifidobacteria strains have reported wide differences regarding the preference and consumption of oligosaccharides and their monomeric components. *Bifidobacterium lactis* grew better in raffinose than in lactose, and *Bifidobacterium longum* used preferably lactose over glucose when provided both as a carbon source (Trindade et al., 2003). Perrin et al. (2001) observed that biomass production of *B. infantis* ATCC 15697 was similar in sucrose and glucose; while Amaretti et al. (2007) observed that the cellular yield of *Bifidobacterium adolescentis* MB 239 was highest when growing in galactose, followed by lactose and GOS, and the lowest in the presence of glucose.

The growth rate of *Bifidobacterium* sp. JCLA3 was highest when using glucose, followed by sucrose, lactose and inulin. However, the adaptation phase to the glucose medium was higher, followed by sucrose, lactose and inulin. The adaptation phase for glucose was 3 h more than the other three substrates. Specific growth rates reported for other bifidobacteria strains are better in complex substrates than in their monomeric units (Gibson and Wang, 1994; Hopkins et al., 1998; Rada et al., 2002; Kim et al., 2003; Palframan et al., 2003; Van der Meulen et al., 2004; Amaretti et al., 2006, 2007).

A wide variety of results have been observed for the utilization of carbon sources by bifidobacteria. In some cases, monosaccharides are mainly used over oligosaccharides (Mlobeli et al., 1998; Van der Meulen et al., 2006); whereas in other species, cellular performances are better in disaccharides and oligosaccharides than in monosaccharides (Kim et al., 2003; Amaretti et al., 2006; Hopkins et al., 1998). *B. lactis* grew better in raffinose than in lactose, and *B. longum* used lactose preferably over glucose when both were available as carbon sources (Trindade et al., 2003). These results can be directly related to the transport systems and their major efficiency for oligosaccharides than for the monomeric components (Trojanova et al., 2006).

The fermentation of prebiotics by bifidobacteria may promote some specific physiological functions through the release of metabolites from the bacteria, especially short chain fatty acids (acetate, propionate and butyrate) and organic acids (lactic acid) into the lumen of the intestine. Short chain fatty acids (SCFAs) may act directly or indirectly (by modifying the pH) on intestinal cells and may be involved in the control of various processes such as the proliferation of mucosa, inflammation, colorectal, carcinogenesis, mineral absorption and the elimination of nitrogenated compounds. Also, SCFAs stimulate sodium and water adsorption in the colon and are known for their ability to induce enzymes that promote mucosal restitution (D'Argenio and Mazzacca, 1999). In addition, lactic acid has an inhibitory effect on *Salmonella enterica* (Makras et al., 2006).

The ability of *bifidobacterium* to survive in acidic conditions depends on the species and source. Matzumoto et al. (2004) reported the viability of *B. longum* and *B. adolescentis* at pH 3.0. Charteris et al. (1998) observed a resistance to acidic conditions for 90 min in *B. bifidum, Bifidobacterium animalis, Bifidobacterium breve and B. infantis*. Probiotic bifidobacteria decreased in numbers very slowly in frozen storage, but are known to die more rapidly at refrigeration temperatures (4°C) (Maus and Ingham, 2003), possibly as a result of sensitivity to oxygen (Shimamura et al., 1992).

Bifidobacterium bifidum was found to be tolerant to the acidity of a gastrointestinal tract system model, with only a 20% decrease in numbers as the pH decreased from 5.0 to 1.8 over an 80-min period (Martteau et al., 1997). Generally, *Bifidobacterium* have been subjected to sublethal acid stress conditions and little has been reported on prolonged acid exposure. When the bacteria were expo-sed to acidic conditions, the homeostatic pH was main-tained by releasing the cell H^+. The F_1F_0-ATPase proton pump was responsible for the survival of some organisms in acidic environments (Cotter and Hill, 2003). Furthermore, this system was dependent on the activity of the ATPase enzyme responsible for maintaining the concentration of H^+ between the cell and environment (Matzumoto et al., 2004; Saarela et al., 2004).

The expression levels of both genes (*ack* and *ldh*) were higher while growing on glucose and inulin, suggesting that production of acetate and lactate were favored in these carbon sources. The reason for the low expression levels of the *ack* gene may be because in the bifidus shunt, acetate is produced in two points of the pathway (Suzuki et al., 2010) and the acetate kinase enzymes could be different; Genbank reports at least two different sequences for acetate kinases in the genus *Bifidobacterium*.

In previous studies with bifidobacteria, it has been demonstrated that *B. longum* NCC2705 growing on MRS medium with different monosaccharides as carbon sources, has its major production of acetic acid using glucose, nevertheless, this production did not measure up in other more complex sources of carbon (Liu et al., 2011). Comparative studies of the growth of *B. adolescentis* MB 239 in SM medium with glucose, galactose, lactose and GOS as carbon sources, showed a major production of acetic acid in GOS (280.6 mM) regarding other substrates: 182.3, 218.6, 202.8 mM, respectively (Amaretti et al., 2007). The inhibitory effects of bifidobacteria against various pathogens including *E. coli, Shigella dysenteriae* and *Yersinia enterocolitica* were demonstrated by numerous *in vitro* studies, and the mechanism of inhibition could be related to the production of acetic and lactic acids (Biavati et al., 2000).

Conclusions

The isolation of an acid-resistant *Bifidobacterium* strain from human faeces is important to know the microbiota of a given population. The strain could be used as a probiotic to improve human health by protecting the host from enteropathogenic infections by the high levels of *ack* and *ldh* expression. The production of acetic and lactic acids as the main metabolites of the bifidus shunt improves intestinal defence mediated by epithelial cells and, thereby, protects the host against lethal infection. It has been demonstrated at the molecular level that acetate inhibits the translocation of a toxin of *E. coli* from the gut lumen to the blood and that lactic acid has an inhibitory effect over other enteropathogenic strains.

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