Catabolism of Glucose and Lactose in *Bifidobacterium animalis* subsp. *lactis*, Studied by ^{13}C Nuclear Magnetic Resonance

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Bifidobacteria are widely used as probiotics in several commercial products; however, to date there is little knowledge about their carbohydrate metabolic pathways. In this work, we studied the metabolism of glucose and lactose in the widely used probiotic strain *Bifidobacterium animalis* subsp. *lactis* BB-12 by *in vivo* ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The metabolism of $[1-^{13}\text{C}]$ glucose was characterized in cells grown in glucose as the sole carbon source. Moreover, the metabolism of lactose specifically labeled with ^{13}C on carbon 1 of the glucose or the galactose moiety was determined in suspensions of cells grown in lactose. These experiments allowed the quantification of some intermediate and end products of the metabolic pathways, as well as determination of the consumption rate of carbon sources. Additionally, the labeling patterns in metabolites derived from the metabolism of glucose specifically labeled with ^{13}C on carbon 1, 2, or 3 in cells grown in glucose or lactose specifically labeled in carbon 1 of the glucose moiety ($[1-^{13}\text{C}]_{\text{glucose}}$ lactose), lactose specifically labeled in carbon 1 of the galactose moiety ($[1-^{13}\text{C}]_{\text{galactose}}$ lactose), and $[1-^{13}\text{C}]$ glucose in lactose-grown cells were determined in cell extracts by ^{13}C NMR. The NMR analysis showed that the recovery of carbon was fully compatible with the fructose 6-phosphate, or bifid, shunt. The activity of lactate dehydrogenase, acetate kinase, fructose 6-phosphate phosphoketolase, and pyruvate formate lyase differed significantly between glucose and lactose cultures. The transcriptional analysis of several putative glucose and lactose transporters showed a significant induction of Balat_0475 in the presence of lactose, suggesting a role for this protein as a lactose permease. This report provides the first *in vivo* experimental evidence of the metabolic flux distribution in the catabolic pathway of glucose and lactose in bifidobacteria and shows that the bifid shunt is the only pathway involved in energy recruitment from these two sugars. On the basis of our experimental results, a model of sugar metabolism in *B. animalis* subsp. *lactis* is proposed.

Bifidobacteria are one of the main microbial groups present in the gastrointestinal tract (GIT) of mammals, and some strains of this genus are frequently used as probiotics (1). Strains of *Bifidobacterium animalis* subsp. *lactis*, such as BB-12, are widely used commercially, and they are included in several probiotic products in a variety of foods and dietary supplements. Several studies have suggested that health-promoting effects can be attributed to this strain (2–5).

Early evidence suggests that in bifidobacteria, monosaccharides are metabolized by the so-called fructose 6-phosphate, or bifid, shunt (Fig. 1) (6–9). The fructose 6-phosphate phosphoketolase (Xfp) is the characteristic enzyme of this path, which holds a dual substrate specificity, acting on fructose 6-phosphate or xylulose 5-phosphate to produce aldose phosphate, acetyl phosphate, and H_2O (10–12). Glyceraldehyde 3-phosphate and acetyl phosphate are further metabolized to produce the end metabolites of the pathway, with acetate, lactate, and ethanol being the most abundant (13).

Various studies have shown the preference of some *Bifidobacterium* species for di- or oligosaccharides over monosaccharides (14–16). In this context, the *Bifidobacterium adolescentis*-specific growth rate was higher in lactose (Lac) than in glucose (Glc) (15, 17), and *Bifidobacterium longum* NCC2705 preferentially uses Lac over Glc as a carbon source when grown in the presence of both sugars (14). The authors also found the *glcP-ptsG* locus to be involved in the transport of Glc into the cell, with *glcP* being downregulated by Lac (14). On the other hand, the genome sequence of *B. longum* NCC2705 showed the presence of four putative Lac transporters, with none of them being downregulated in the presence of Glc (18). These results suggest an adaptation of some *Bi-*

fidobacterium species to Lac, a disaccharide which is the main sugar ingested by breast-fed infants.

There are relatively few publications dealing with the metabolism of sugars in *Bifidobacterium*. The bifid shunt has been partially characterized in terms of enzymatic activities (9, 19), and the labeling pattern of end products derived from Glc metabolism in *Bifidobacterium bifidum* ATCC 29521 was investigated by ^{13}C nuclear magnetic resonance (NMR) using as metabolic tracers Glc specifically labeled on carbons 1 and 3 (20). *In vivo* ^{13}C NMR is a noninvasive technique that allows the monitoring, online and in real time, of the concentrations of end products and intracellular metabolites, as well as the rates of substrate consumption, during the metabolism of labeled substrate by nongrowing cells under controlled temperature and atmosphere conditions (21). This technique has facilitated the study of different pathways in various species, such as *Lactococcus lactis*, *Staphylococcus aureus*, and *Escherichia coli* (22–24). In this work, we used *in vivo* ^{13}C NMR to characterize the metabolism of $[1-^{13}\text{C}]$ Glc in Glc-grown cells and Lac specifically labeled in carbon 1 of the Glc moiety ($[1-^{13}\text{C}]_{\text{Glc}}$ Lac) and Lac specifically labeled in carbon 1 of the galactose

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FIG 1 Scheme of central carbon metabolism in *B. animalis* subsp. *lactis* BB-12. The putative pathways for Glc, Lac, and galactose metabolism are depicted. End products and intermediate products detected in this work are shown in boxes with dashed borders. Enzymes involved in Glc, Lac, and galactose metabolism are shown in gray. Abbreviations: β -Gal, β -galactosidase; Pgm, phosphoglucomutase; GlkA, glucokinase; Gpi, Glc 6-phosphate isomerase; Xfp, fructose 6-phosphate phosphoketolase; Adh2, alcohol dehydrogenase; AckA, acetate kinase; Ldh2, lactate dehydrogenase; Pfl, pyruvate formate lyase; Tal, transaldolase; Tkt, transketolase; GalK, galactokinase; GalT, galactose 1-phosphate uridylyltransferase; UgpA, UTP-Glc 1-phosphate uridylyltransferase; GalE, UDP-Glc 4-epimerase; Gap, glyceraldehyde 3-phosphate dehydrogenase; Pgk, phosphoglycerate kinase; Gpm, phosphoglycerate mutase; Eno, enolase; Pyk, pyruvate kinase; Glc, glucose; Gal, galactose; Gal1P, galactose 1-phosphate; Glc1P, glucose 1-phosphate; Glc6P, glucose 6-phosphate; F6P, fructose 6-phosphate; E4P, erythrose 4-phosphate; S7P, sedoheptulose 7-phosphate; R5P, ribulose 5-phosphate; X5P, xylulose 5-phosphate; GAP, glyceraldehyde 3-phosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Lactate_{Out}, extracellular lactate; Acetate_{Out}, extracellular acetate.

(Gal) moiety ($[1-^{13}\text{C}_{\text{Gal}}]\text{Lac}$) to unveil the fate of the two Lac moieties in Lac-grown cells in the commonly used probiotic strain *B. animalis* subsp. *lactis* BB-12. Moreover, we used perchloric acid cell extract experiments to confirm that Glc and Lac metabolism in BB-12 goes through by the bifid shunt. For this pur-

pose, we characterized the metabolism of $[1-^{13}\text{C}]\text{Glc}$, $[2-^{13}\text{C}]\text{Glc}$, and $[3-^{13}\text{C}]\text{Glc}$ in Glc-grown cells and we used $[1-^{13}\text{C}_{\text{Glc}}]\text{Lac}$, $[1-^{13}\text{C}_{\text{Gal}}]\text{Lac}$, and $[1-^{13}\text{C}]\text{Glc}$ in Lac-grown cells to unravel the partitioning of sugar substrate carbon throughout the metabolic network of this strain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strain *B. animalis* subsp. *lactis* BB-12 was grown in MRS fermentation broth, which contains peptone (10 g liter⁻¹), yeast extract (10 g liter⁻¹), meat extract (5 g liter⁻¹), K₂HPO₄ (2 g liter⁻¹), sodium acetate (5 g liter⁻¹), ammonium citrate (2 g liter⁻¹), magnesium sulfate (0.2 g liter⁻¹), manganese sulfate (0.05 g liter⁻¹), and 0.1% (vol/vol) Tween 80 (MRSfc) supplemented with 2% (wt/vol) Glc, galactose, or Lac as a carbon source and 0.25% (wt/vol) L-cysteine. Extracts, peptone, chemicals, and supplements were obtained from Sigma Chemical Co. (St. Louis, MO). Cells were grown at 37°C under anaerobic conditions in bottles sealed with rubber stoppers. In order to obtain proper anaerobic conditions, the growth medium was previously reduced by bubbling argon for 20 min before sterilization. Bottles were inoculated through the rubber stoppers with the help of a needle, without disrupting the anaerobic atmosphere within the bottles. Growth was evaluated by measuring the turbidity of the culture by determination of the optical density at 600 nm (OD₆₀₀). Inocula were prepared to an initial OD₆₀₀ of about 0.25. Growth was followed, and cells were harvested during the mid-exponential growth phase (OD₆₀₀ about 1). The maximum specific growth rate ($\mu_{\max}$) was calculated from the slope obtained through linear regressions of the plots of ln OD₆₀₀ versus time during the exponential growth phase.

In vivo ¹³C NMR experiments. Cells grown to exponential phase were centrifuged, washed twice, and suspended to a concentration at an OD₆₀₀ of approximately 100 (dry weight of about 25 to 35 mg liter⁻¹) in 100 mM KP_i (pH 6.5) with 6% (vol/vol) D₂O. *In vivo* NMR experiments were performed using a 10-mm NMR tube containing 3 ml of cell suspension. To avoid the cells settling and to ensure an adequate supply of gases to the cell suspension, an airlift system was used inside the NMR tube (25). To make the system anaerobic, argon was bubbled through the airlift system 10 min before and continuously after acquisition was started. Glc (20 mM) specifically labeled in carbon 1, was added to a suspension of cells grown in MRSfc with Glc as the carbon source. Lac (15 mM) specifically labeled in carbon 1 of the Glc moiety or Lac (15 mM) specifically labeled in carbon 1 of the galactose moiety was added to suspensions of cells grown in MRSfc with Lac as the carbon source. Spectra were acquired at time point zero and sequentially (every 30 s) after substrate addition. When the substrate was exhausted and no changes in the resonances of the end products were observed, a perchloric acid cell extract was prepared as described previously (21). The cell extract was neutralized to pH 6.5 with potassium hydroxide, and the potassium perchlorate precipitate, as well as cell debris and denatured macromolecules, were removed by centrifugation. The supernatant (NMR extract) was used for quantification of end products and minor metabolites as described below. For dry weight determination, cells were harvested by filtration through 0.22- μ m-pore-size membranes and dried to a constant weight at 100°C. Each type of *in vivo* NMR experiment was repeated at least three times, and the results were highly reproducible.

Quantification of products by NMR. The lactate, acetate, and ethanol in NMR extracts were quantified by ¹H NMR (21). Formic acid (sodium salt) was added to the samples and used as an internal concentration standard. The amounts of end products and other labeled metabolites were determined from the analysis of the ¹³C spectra of the NMR extracts, as described by Neves et al. (21). The concentration of labeled lactate determined by ¹H NMR was used as a standard to calculate the concentration of the other metabolites in the sample.

NMR spectroscopy. Carbon 13 spectra were acquired at 125.77 MHz on a Bruker Avance II 500-MHz spectrometer (Bruker Bio-Spin GmbH, Karlsruhe, Germany). All *in vivo* experiments were run using a quadrupole nucleus probe head at 37°C, as described elsewhere (21). The acquisition parameters were as follows: spectral width, 30 kHz; pulse width, 9 μ s (60° flip angle); data size, 32K; recycle delay, 1.5 s; and number of transients, 20. Carbon chemical shifts are referenced to the resonance of external methanol, which was designated at 49.3 ppm.

Glc and Lac fermentation pathway in BB-12. The metabolism of 20 mM [^{1-¹³C}]Glc, [^{2-¹³C}]Glc, and [^{3-¹³C}]Glc was studied in cell suspensions of strain BB-12 grown in MRSfc with 2% (wt/vol) Glc under the conditions described above. The metabolism of 15 mM [^{1-¹³C}]_{Glc}Lac, [^{1-¹³C}]_{Gal}Lac, and [^{1-¹³C}]Glc was studied in suspensions of cells grown in MRSfc with 2% Lac. Samples (1 ml) were collected at different time points after the addition of the labeled carbon source (0, 2.5, and 60 min). A perchloric acid cell extract of each collected sample was prepared as described above and stored at -20°C until further analysis by ¹H and ¹³C NMR for quantification of end products and other labeled metabolites, as described above. The values reported are averages of at least two independent experiments. For the calculation of the correction factors and the metabolite concentrations in cell extracts, ¹³C NMR spectra were acquired with a 60° flip angle and a recycle delay of 1.5 s (under saturating conditions) or 60.5 s (under relaxed conditions). Spectra were analyzed using TopSpin software (Bruker Corporation). The correction factors were determined for formate (1.25 $\pm$ 0.05), ethanol (0.99 $\pm$ 0.23), lactate (0.98 $\pm$ 0.09), and acetate (0.84 $\pm$ 0.18).

Enzyme activity measurements. The enzyme activities of some bifid shunt enzymes were determined. Cells were harvested at mid-exponential growth phase, washed twice with 100 mM Tris-HCl (pH 6.5), and resuspended in 50 mM Tris-HCl buffer (pH 6.5) to a final OD of about 80 to 120. All enzymes were assayed after mechanical cell lysis by passage twice through a One-Shot cell disrupter at 2.1 kilobars (Constant Systems Ltd., Daventry, United Kingdom) and centrifugation for 15 min at 30,000 $\times$ g to remove cell debris, except for the pyruvate formate lyase (Pfl) activity, for which the cells were disrupted under anaerobic conditions by vortexing them three times with glass beads (200 mg; diameter, 0.1 mm; Sartorius AG, Goettingen, Germany) for 120 s; in between the vortexing procedures, the suspensions were kept on ice. All enzymatic reactions were performed under strict anaerobic conditions. The protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Pfl activity was determined in the direction of pyruvate formation. The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.5), 10 mM MgSO₄, 750 mM acetate, 750 mM acetyl phosphate, 150 mM formate, and 150 mM acetyl coenzyme A (acetyl-CoA). The mixture was incubated at 37°C, and the reaction was stopped at different time points (0, 5, 10, 20, 40 min) by incubation at 80°C for 2 min. The supernatants were analyzed by high-performance liquid chromatography. Specific activity was expressed as units (μ mol min⁻¹) per milligram of protein [U (mg of protein)⁻¹], with 1 unit of enzyme activity being the amount of protein catalyzing the formation of 1 μ mol of pyruvate per minute under the experimental conditions used.

Fructose 6-phosphate phosphoketolase (Xfp) activity was measured spectrophotometrically as the amount of ferric acetyl hydroxamate produced from the enzymatically generated acetyl phosphate, as described previously (26). Quantification was carried out by comparisons with a series of acetyl phosphate standards. One unit of activity was defined as the amount of protein that released 1 μ mol of acetyl phosphate per min. Specific activity was expressed as U (mg of protein)⁻¹. Lactate dehydrogenase (Ldh) was assayed as described previously (27), with some modifications. Ldh activity was assayed in a reaction mixture consisting of 100 mM Tris-HCl (pH 7.2), 5 mM MgCl₂, 150 mM fructose 6-phosphate, 400 mM pyruvate, 15 mM NADH, and 20 μ g of total bacterial protein. Specific activity was expressed as U (mg of protein)⁻¹, with 1 unit of enzyme activity being the amount of enzyme catalyzing the conversion of 1 μ mol of substrate per minute under the experimental conditions used.

Acetate kinase (AckA) activity was assayed as described previously (28). The reaction was carried out with 20 μ g of total bacterial protein per assay. One unit of activity was defined as the amount of protein producing 1 μ mol of hydroxamate per min, and the specific activity was expressed as U (mg of protein)⁻¹.

Results were expressed as the means of three independent growth experiments. Results were analyzed using SPSS software (SPSS Inc., Chicago, IL). The enzymatic activities assayed in Glc-grown cells were com-

FIG 2 Growth profile of *B. animalis* subsp. *lactis* BB-12 in MRSfc broth with Lac (A) or with Glc (black circles) or galactose (white squares) (B) as the carbon source at 37°C under anaerobic conditions. At the times indicated by the arrows, a culture sample was withdrawn for *in vivo* NMR analysis. Data are from three independent experiments.

pared with those assayed in Lac-grown cells by using analysis of variance (ANOVA) with two factors, Glc and Lac.

qRT-PCR experiments. Quantitative reverse transcription-PCR (qRT-PCR) was used to determine the expression of genes coding for putative Glc and Lac transporters in strain BB-12. Cells were grown to

mid-exponential phase as indicated above. The protocols for cell lysis, RNA isolation, and cDNA synthesis were performed as previously described (29). Specific primers (see Table 2) were designed with Primer Express software (Applied Biosystems, Foster City, CA) on the basis of the complete genome of *B. animalis* subsp. *lactis* DSM 10140 (GenBank ac-

FIG 3 $[1-^{13}\text{C}]$ Lac metabolism in anaerobically grown *B. animalis* subsp. *lactis* BB-12 monitored by *in vivo* ^{13}C NMR. (A) Sequence of the ^{13}C NMR spectra acquired during the metabolism of 15 mM $[1-^{13}\text{C}]$ Lac by a cell suspension of BB-12 under anaerobic conditions at 37°C. Each spectrum represents 30 s of acquisition. Lac was added at time zero, and each spectrum was acquired during the indicated interval and processed with a 2-Hz line broadening. (B) Kinetics of $[1-^{13}\text{C}]$ Lac (15 mM) consumption and end product formation. The maximal Lac consumption rates ($\mu\text{mol min}^{-1} \text{mg} [\text{dry weight}]^{-1}$) are indicated in a box in the upper-right corner. Symbols: pink square, $[1-^{13}\text{C}]$ Lac; black diamond, Glc; orange square, extracellular lactate; green triangle, extracellular acetate; purple diamond, total ethanol. The result of a representative experiment is depicted. For the sake of clarity, data for only the first 30 min of Lac metabolism are represented; therefore, values corresponding to later time points indicated in the text do not match with the values in the figure. Intracellular metabolite concentrations were not corrected by the intracellular volume, and thus, the concentration values are presented as if the metabolite was extracellular. Abbreviations: Lac, lactose; Glc, glucose; Act_{out} , extracellular acetate; Lct_{out} , extracellular lactate; EtOH, total ethanol.

FIG 4 $[1-^{13}\text{C}_{\text{Gal}}]\text{Lac}$ metabolism in anaerobically grown *B. animalis* subsp. *lactis* BB-12 monitored by *in vivo* ^{13}C NMR. (A) Sequence of ^{13}C NMR spectra acquired during the metabolism of 15 mM $[1-^{13}\text{C}_{\text{Gal}}]\text{Lac}$ by a cell suspension of BB-12 under anaerobic conditions at 37°C. Each spectrum represents 30 s of acquisition. Lac was added at time zero, and each spectrum was acquired during the indicated interval and processed with a 2-Hz line broadening. (B) Kinetics of $[1-^{13}\text{C}_{\text{Gal}}]\text{Lac}$ (15 mM) consumption and end product formation. The maximal Lac consumption rate ($\mu\text{mol min}^{-1} \text{mg} [\text{dry weight}]^{-1}$) is indicated in a box in the upper-right corner. Symbols: blue square, $[1-^{13}\text{C}_{\text{Gal}}]\text{Lac}$; gray diamond, galactose; orange square, extracellular lactate; green triangle, extracellular acetate. The result of a representative experiment is depicted. For the sake of clarity, data for only the first 30 min of Lac metabolism are represented; therefore, values corresponding to later time points indicated in the text do not match with the values in the figure. Intracellular metabolite concentrations were not corrected by the intracellular volume, and thus, the concentration values are presented as if the metabolite was extracellular. Abbreviations: Lac, lactose; Gal, galactose; Act_{out} , extracellular acetate; Lct_{out} , extracellular lactate.

cession number CP001606.1). Primers for putative Lac transporters were designed for genes adjacent to β -galactosidase-encoding genes, previously annotated to be potential carbohydrate transporters in the genome of *B. animalis* subsp. *lactis* DSM 10140. Primers for putative Glc transporters were designed for genes *glcU* and *glcP*, previously described to be potential Glc transporters in *B. animalis* subsp. *lactis* DSM 10140 and *B. longum* NCC2705, respectively (14, 30).

The qRT-PCR experiments were performed as described by Gueimonde et al. (31). Samples from two biological replicates were analyzed in at least two independent PCR runs. Results were analyzed using SPSS software.

RESULTS

Growth of *B. animalis* subsp. *lactis* BB-12 on Lac and its constituent monosaccharides. The growth of *B. animalis* subsp. *lactis* BB-12 on different sugar substrates has been reported previously (32). In this work, strain BB-12 was grown in our MRSfc supplemented with 2% (wt/vol) Lac, Glc, or galactose to establish the representative growth profiles, which enabled standardization in samples harvested for further metabolic studies (Fig. 2). Galactose was a very poor substrate for growth and thus not further used as

a growth substrate. In Lac or Glc, maximal OD_{600} s of 8.2 ± 0.9 and 8.3 ± 0.9 , respectively, were obtained, with the cells growing at maximum specific growth rates of 0.38 ± 0.03 and $0.40 \pm 0.03 \text{ h}^{-1}$, respectively. For the metabolic studies described below, cells were harvested at an OD_{600} of 1, as this point is considered mid-exponential growth phase for growth on both Lac and Glc (Fig. 2).

Metabolism of Lac monitored by *in vivo* ^{13}C NMR. (i) **Metabolic fate of Glc moiety in Lac: catabolism of $[1-^{13}\text{C}_{\text{Glc}}]\text{Lac}$.** Figure 3A shows a typical sequence of the sugar anomeric carbon region of ^{13}C spectra acquired during the anaerobic metabolism of $[1-^{13}\text{C}_{\text{Glc}}]\text{Lac}$ (15 mM) by nongrowing suspensions of BB-12. After addition of $[1-^{13}\text{C}_{\text{Glc}}]\text{Lac}$, besides the two resonances due to the β and α forms of the Glc moiety in Lac (δ , 96.08 and 92.15 ppm, respectively), two additional resonances (δ , 96.23 and 92.41 ppm) assigned to the β and α forms of the monosaccharide Glc labeled in carbon 1, respectively, were immediately detected. Two resonances that appeared downfield of β - and α -Glc (δ , 96.35 and 92.51 ppm, respectively) were assigned to β -Glc 6-phosphate and α -Glc 6-phosphate, respectively.

FIG 5 [1-¹³C]Glc metabolism in anaerobically grown *B. animalis* subsp. *lactis* BB-12 monitored by *in vivo* ¹³C NMR. (A) Kinetics of [1-¹³C]Glc (20 mM) consumption, end product formation, and phosphorylated intermediates. The maximal Glc consumption rate ($\mu\text{mol min}^{-1} \text{mg} [\text{dry weight}]^{-1}$) is indicated in a box in the upper-right corner. (B) Dynamics of intra- and extracellular pools of lactate and acetate; the results for Glc are also shown for the sake of clarity. Symbols: black diamond, Glc; orange square, total lactate; green triangle, total acetate; purple diamond, total ethanol; open orange square, extracellular lactate; gray circle, intracellular lactate; brown circle, extracellular acetate; open green triangle, intracellular acetate. Total lactate and total acetate are the sum of the extra- and intracellular concentrations without correction of the latter for the internal volume. The result of a representative experiment is depicted. For the sake of clarity, only the data for the first 30 min of Glc metabolism are represented; therefore, values corresponding to later time points indicated in the text do not match with the values in the figure. Intracellular metabolite concentrations were not corrected by the intracellular volume, and thus, the concentration values are presented as if the metabolite was extracellular. Abbreviations: Glc, glucose; Act, acetate; Lct, lactate; EtOH, total ethanol; Act_{in}, intracellular acetate; Act_{out}, extracellular acetate; Lct_{in}, intracellular lactate; Lct_{out}, extracellular lactate.

The kinetics of [1-¹³C_{Glc}]Lac consumption, end product formation, and intracellular metabolite pools are shown in Fig. 3B. Under the conditions tested (anaerobic, without pH control) the consumption of [1-¹³C_{Glc}]Lac displayed biphasic kinetics: a first faster and linear phase ($0.12 \pm 0.04 \mu\text{mol min}^{-1} \text{mg} [\text{dry weight}]^{-1}$) was followed by a slower and nonlinear phase. During the fast phase of [1-¹³C_{Glc}]Lac, Glc peaked at a maximal concentration of $4.1 \pm 0.8 \text{ mM}$ and was subsequently consumed at an initial rate of $0.02 \pm 0.004 \mu\text{mol min}^{-1} \text{mg} [\text{dry weight}]^{-1}$ that decreased progressively (probably due to acidification of the medium). The buildup of the [1-¹³C]Glc 6-phosphate pool was observed soon after the addition of [1-¹³C_{Glc}]Lac; its concentration then peaked and declined to levels below detection following [1-¹³C]Glc consumption.

[2-¹³C]acetate was the main end product of [1-¹³C_{Glc}]Lac metabolism, but minor amounts of [3-¹³C]lactate ($0.4 \pm 0.2 \text{ mM}$) and [2-¹³C]ethanol ($0.6 \pm 0.1 \text{ mM}$) were also detected. The carbon recovery from [1-¹³C_{Glc}]Lac metabolism was $95.0\% \pm 1.1\%$.

The utilization of ¹H NMR for the quantification of end products in cell extracts enables the determination of both ¹³C-labeled and unlabeled fractions of end products. The concentrations of unlabeled end products derived from 15 mM [1-¹³C_{Glc}]Lac were as follows: acetate, $36.2 \pm 3.2 \text{ mM}$; lactate, $19.9 \pm 1.4 \text{ mM}$; ethanol, $3.0 \pm 2.0 \text{ mM}$; and formate, $5.2 \pm 1.9 \text{ mM}$. The acetic acid/lactic acid ratio for this condition was 2.4 ± 0.04 .

(ii) Metabolic fate of galactose moiety in Lac: catabolism of [1-¹³C_{Gal}]Lac. A sequence of the sugar anomeric carbon region of the ¹³C spectra acquired during the anaerobic metabolism of [1-¹³C_{Gal}]Lac (15 mM) by nongrowing suspensions of BB-12 is shown in Fig. 4A. Resonances due to the β and α forms of [1-¹³C_{Gal}]Lac appeared at δ values of 103.18 and 103.16 ppm, respectively. Two resonances at δ values of 96.76 and 92.58 ppm were assigned to the β and α forms of [1-¹³C]Gal (pyranose conformations), respectively. Weaker resonances at δ values of 101.7 and 95.6 ppm corresponded to the furanose conformations of [1-¹³C]Gal. In a few spectra, a faint resonance due to α -Glc 1-phosphate (δ , 94.15 ppm), an intermediate of the Leloir pathway, was

also visible. However, reliable quantification of this resonance was not possible. Overall, our data are in good agreement with the initial steps proposed in the literature for Lac uptake in bifidobacteria: uptake via a Lac permease followed by β -galactosidase cleavage to Glc and galactose.

As expected, the profile of [1-¹³C_{Gal}]Lac consumption fully resembled that of [1-¹³C_{Glc}]Lac consumption, displaying biphasic kinetics and similar values for the maximal rate of utilization (Fig. 4; compare Fig. 4 to Fig. 3). In a fashion similar to that for the [1-¹³C]Glc derived from the [1-¹³C_{Glc}]Lac, the free [1-¹³C]Gal derived from [1-¹³C_{Gal}]Lac increased to a maximal concentration of $4.4 \pm 0.5 \text{ mM}$, decreasing slowly after the switch to slow Lac utilization. Interestingly, the rate of [1-¹³C]Gal depletion ($<0.01 \mu\text{mol min}^{-1} \text{mg} [\text{dry weight}]^{-1}$) was about 3 times lower than that of [1-¹³C]Glc, indicating a limitation of BB-12 in its capacity to utilize galactose. This metabolic impairment is consistent with the reduced ability of the strain to grow in galactose. The pool of [2-¹³C]acetate increased immediately after substrate addition, reaching a maximal concentration of $12.8 \pm 0.2 \text{ mM}$. [3-¹³C]lactate accumulated to a concentration of $0.5 \pm 0.1 \text{ mM}$, while the concentration of [2-¹³C]ethanol was below the detection limit of the *in vivo* ¹³C NMR. A value of about 0.4 mM was measured in cell extracts. The carbon recovery from [1-¹³C_{Gal}]Lac metabolism was $99.4\% \pm 6.08\%$.

The unlabeled end products derived from 15 mM [1-¹³C_{Gal}]Lac detected by ¹H NMR in the cell extract were as follows: acetate, $38.4 \pm 2.3 \text{ mM}$; lactate, $22.2 \pm 2.9 \text{ mM}$; ethanol, $2.6 \pm 0.6 \text{ mM}$; and formate, $3.4 \pm 0.6 \text{ mM}$. The acetic acid/lactic acid ratio for this condition was 2.3 ± 0.2 .

Metabolism of [1-¹³C]Glc monitored by *in vivo* ¹³C NMR. The kinetics of [1-¹³C]Glc (20 mM) consumption, end product formation, and intracellular metabolite pools are shown in Fig. 5. After the addition of [1-¹³C]Glc to the cell suspension, resonances due to the β and α Glc forms (δ , 96.2 and 92.4 ppm, respectively) of the sugar were detected and progressively decreased until the Glc was exhausted (data not shown). Under the conditions exam-

TABLE 1 Metabolite concentrations in perchloric extracts from cell suspensions of *B. animalis* subsp. *lactis* BB-12^a

Cell and substrate	Substrate isotopomer	Mean ± SD metabolite concn (mM)							
		Acetate					Lactate		
		[1,2- ¹³ C]acetate	[1- ¹³ C]acetate	[2- ¹³ C]acetate	Total acetate	Total by NMR	[2,3- ¹³ C]lactate	[2- ¹³ C]lactate	[3- ¹³ C]lactate
Glc (20 mM)-grown cells									
[1- ¹³ C]Glc	0.66 ± 0.16		0.08 ± 0.11	16.80 ± 0.42	22.45 ± 0.64	39.33 ± 1.17			0.88
[2- ¹³ C]Glc			18.15 ± 0.49	0.21 ± 0.21	21.5 ± 0.42	39.86 ± 0.28		0.17 ± 0.03	0.04 ± 0.03
[3- ¹³ C]Glc		8.85 ± 0.78	0.55 ± 0.07	0.55 ± 0.07	27.4 ± 0.28	37.3 ± 0.42	0.2		
Lac (15 mM)-grown cells									
[1- ¹³ C _{Glc}]Lac	0.44 ± 0.62			12.2 ± 0.42	40.45 ± 1.34	52.65 ± 1.77			0.13 ± 0.01
[1- ¹³ C _{Gal}]Lac	1.05 ± 0.13			10.85 ± 0.64	32.45 ± 0.21	43.30 ± 0.85			0.10 ± 0.03
[1- ¹³ C]Glc	0.35 ± 0.29			11.25 ± 0.64	18.00 ± 0.85	29.25 ± 1.48			0.24 ± 0.03

^a *B. animalis* subsp. *lactis* BB-12 was grown in MRSfc plus Glc after one pulse of 20 mM [1-¹³C]Glc, [2-¹³C]Glc, or [3-¹³C]Glc or grown in MRSfc plus Lac after one pulse of 15 mM [1-¹³C_{Glc}]Lac, [1-¹³C_{Gal}]Lac, or [1-¹³C]Glc, as quantified by ¹³C NMR and ¹H NMR. Values represent means ± standard deviations from duplicate assays. EtOH, ethanol; Glc-6-P, Glc 6-phosphate; Gal-1-P, α-galactose 1-phosphate.

ined (anaerobic, without pH control), Glc consumption was biphasic, displaying a fast and linear phase (above 2.5 mM), followed by a slow, nonlinear pattern of utilization (Fig. 5). During the fast phase, Glc was consumed at a rate of 0.18 ± 0.07 μmol min⁻¹ mg (dry weight)⁻¹.

In the ¹³C NMR spectra of BB-12 cell suspensions metabolizing [1-¹³C_{Glc}]Lac, two resonances were assigned to the methyl group of intracellular and extracellular acetate (data not shown). The pH gradient between the cytoplasm and the external medium is expected to originate a considerable difference in the protonation of intra- and extracellular pools of weak organic acids, which results in the appearance of two distinct resonances in an NMR spectrum (24). Extracellular and intracellular [2-¹³C]acetate pools were detected immediately after substrate addition; the intracellular acetate peaked at the onset of the switch between fast and slow Glc consumption and progressively decreased to a steady level (Fig. 5). The accumulation of acetate (maximal concentration, 18.0 ± 1.4 mM) in the medium mirrored the consumption of Glc but was still observed beyond its exhaustion due to the secretion of the intracellular acetate. Acetate was clearly the main end product from Glc catabolism, but [3-¹³C]lactate (both intracellular and extracellular pools) and [2-¹³C]ethanol were also detected. The extracellular and intracellular lactate reached maximal and transient maximal concentrations of 0.8 ± 0.3 and 0.5 ± 0.2 mM, respectively. Ethanol reached a maximal concentration of 1.0 ± 0.2 mM. Acetyl phosphate was also detected, while Glc was available, decreasing to undetectable levels before Glc exhaustion (data not shown). The maximal concentration of [2-¹³C]acetyl phosphate was 0.5 ± 0.2 mM. The carbon recovery from Glc was 100.6% ± 4.39%. The concentrations of unlabeled end products derived from 20 mM [1-¹³C]Glc were as follows: acetate, 18.2 ± 2.2 mM; lactate, 11.2 ± 0.9 mM; ethanol, 2.7 ± 1.8 mM; and formate, 6.5 ± 1.1 mM. The acetic acid/lactic acid ratio for this condition was 3.0 ± 0.3.

Labeling pattern of end products in cell extracts obtained during the metabolism of sugar substrates specifically labeled in C-1, C-2, or C-3. The *in vivo* ¹³C NMR data clearly showed that Lac is taken up via a nonphosphotransferase permease and, once it is internalized, is split via the action of a β-galactosidase to galactose and Glc. Glc is phosphorylated to Glc 6-phosphate, while galactose is seemingly catabolized via the Leloir pathway. Unfortunately, intermediates of the bifid shunt (or other glycolytic pathways) were not detected *in vivo*, and the utilization of substrates specifically labeled on carbon 1 *per se* did not allow different gly-

colysis types to be distinguished. Thus, we examined by ¹³C NMR perchloric acid extracts obtained during the metabolism of Glc and Lac specifically labeled in the carbons of choice at times of 2.5 (glycolytic intermediates) and 60 (end products) min. The metabolism of [1-¹³C]Glc, [2-¹³C]Glc, and [3-¹³C]Glc was analyzed in BB-12 cells grown on Glc, while nongrowing BB-12 suspensions of cells grown on Lac were used to examine the catabolism of [1-¹³C_{Glc}]Lac, [1-¹³C_{Gal}]Lac, and [1-¹³C]Glc (Table 1). Under the conditions tested (time, 2.5 min), glycolytic intermediates were below the detection limit (below 0.01 μM), except for Glc 6-phosphate, which was also detected *in vivo*. α-Glc 1-phosphate, an intermediate of the Leloir pathway, was also detected.

In accordance with our *in vivo* ¹³C NMR data, [1-¹³C]Glc metabolism in BB-12 yielded [2-¹³C]acetate, [3-¹³C]lactate, and [2-¹³C]ethanol. From [2-¹³C]Glc, Glc-grown BB-12 produced [1-¹³C]acetate, [2-¹³C]lactate, and [1-¹³C]ethanol, while the metabolism of [3-¹³C]Glc produced [1,2-¹³C]acetate, [2,3-¹³C]lactate, and [1,2-¹³C]ethanol. The labeling patterns and the respective concentrations (Table 1) are consistent with operation of the bifid shunt.

For BB-12 cells grown on Lac, the labeled end products obtained for the different sugars specifically labeled on carbon 1 ([1-¹³C_{Glc}]Lac, [1-¹³C_{Gal}]Lac, and [1-¹³C]Glc) were as expected: [2-¹³C]acetate, [3-¹³C]lactate, and [2-¹³C]ethanol.

Carbon recoveries obtained by ¹³C NMR were above 90% for all conditions tested. In summary, the labeled end products obtained corresponded to what was expected for the bifid shunt (Fig. 6).

Enzyme activity measurements. Specific activities of different enzymes (Ldh, AckA, Xfp, and Pfl) were measured in cell extracts of BB-12 cells grown on Glc or Lac.

The enzyme activities from Glc cultures were compared with the activities from Lac cultures. The statistical analyses of the data revealed that the enzymatic activities differed significantly between the Glc and Lac cultures. To confirm the production of pyruvate from acetyl-CoA by pyruvate formate lyase (Pfl), its activity was measured in the direction of pyruvate formation. Cells grown on Glc showed less Ldh activity [10.07 ± 0.72 U (mg protein)⁻¹] than those grown on Lac [13.57 ± 1.72 U (mg protein)⁻¹] (*P* < 0.01). In contrast, cells grown in the presence of Glc showed higher AckA [3.23 ± 0.31 U (mg protein)⁻¹; *P* < 0.05], Xfp [3.68 ± 0.06 U (mg protein)⁻¹; *P* < 0.001], and Pfl [1.97 ± 0.10 U (mg protein)⁻¹; *P* < 0.05] activities than those grown in the presence of Lac [2.62 ± 0.31 U (mg protein)⁻¹, 3.25 ± 0.03 U (mg protein)⁻¹, and 1.55 ± 0.13 U (mg protein)⁻¹, respectively].

Gene expression. The expression of genes coding for putative

TABLE 1 (Continued)

Lactate		Ethanol		Total EtOH		Total by NMR		[1- ¹³ C] formate by total NMR		% carbon recovery by NMR	
Total lactate	Total by NMR	[1,2- ¹³ C]EtOH	[1- ¹³ C]EtOH	[2- ¹³ C]EtOH	Total EtOH	Total by NMR					
9.45 ± 0.49	10.33 ± 0.49			0.99 ± 0.04	1.95 ± 0.07	2.95 ± 0.04	7.45 ± 0.21	0.10 ± 0.04		106.45 ± 2.90	
3.55 ± 0.07	3.74 ± 0.13		0.93 ± 0.80		1.90 ± 1.13	2.83 ± 1.93	14.10 ± 0.71			92.25 ± 2.76	
11.85 ± 0.07	12.05 ± 0.07	0.70 ± 0.14			1.65 ± 0.21	2.35 ± 0.35	6.70 ± 0.14			101.05 ± 0.21	
19.70 ± 0.42	19.83 ± 0.41			0.07 ± 0.03	1.75 ± 0.07	1.82 ± 0.10	6.30 ± 0.28			100.15 ± 7.00	
18.55 ± 0.07	18.65 ± 0.10			0.03	1.40 ± 0.28	1.43 ± 0.28	2.40 ± 0.57		0.05 ± 0.01	92.80 ± 2.83	
5.05 ± 0.35	5.29 ± 0.38			0.54 ± 0.25	2.10 ± 0.14	2.64 ± 0.11	6.65 ± 0.64	0.18 ± 0.15		99.15 ± 6.01	

Glc and Lac transporters by exponentially growing BB-12 cells cultured in the presence of either Glc or Lac was assessed using qRT-PCR. Six genes, selected on the basis of high homologies with sugar transporters, were analyzed. Two are putative Glc transporters

(Balat_1097 [GlcU; putative Glc uptake permease] and Balat_1154 [sugar permease of the major facilitator superfamily]), and four are putative Lac transporters (Balat_0054 [glycoside-pentoside-hexuronide:cation symporter family protein], Balat_0475 [LacS, galacto-

FIG 6 Schematic representation of labeling patterns in end products and pathway intermediates derived from the metabolism of [1-¹³C], [2-¹³C], and/or [3-¹³C]Glc in *B. animalis* subsp. *lactis* BB-12. For the sake of simplicity, the metabolic pathway shown begins with two fructose molecules. The carbons of one fructose molecule are numbered in red, and the carbons of the second fructose molecule are numbered in black. These numbers allow the origin of carbon for each product formed in this metabolic pathway to be followed. The carbons highlighted with red originate from Glc labeled on carbon 1 ([1-¹³C]Glc), the blue carbons are from [2-¹³C]Glc, and the green carbons are from [3-¹³C]Glc. To determine the fate of carbon 1 on Glc, the products with carbons highlighted with red must be followed. To determine the fate of carbon 2 on Glc, the carbons highlighted with blue must be followed; similarly, the carbons highlighted with green trace the label originating from [3-¹³C]Glc. End products (and relevant pathway intermediates) derived from [1-¹³C]Glc, [2-¹³C]Glc, and [3-¹³C]Glc are boxed in red, blue, and green, respectively. The labeled end products from [1-¹³C]Glc are as follows: two [2-¹³C]acetate molecules, one [3-¹³C]lactate molecule, and one [2-¹³C]ethanol molecule. The labeled end products from [2-¹³C]Glc are as follows: two [1-¹³C]acetate molecules, one [2-¹³C]lactate molecule, and one [1-¹³C]ethanol molecule. The labeled end products from [3-¹³C]Glc are as follows: one [1,2-¹³C]acetate molecule, one [2,3-¹³C]lactate molecule, and one [1,2-¹³C]ethanol molecule. Dashed arrow, lower Embden-Meyerhof-Parnas pathway. For abbreviations, see the legend to Fig. 1.

TABLE 2 Expression of selected genes of *B. animalis* subsp. *lactis* BB-12 and primers used for qRT-PCR analysis

Gene	Primer sequence (forward/reverse)	Annotation in the genome of strain DSM 10140	Fold change for Lac/Glc ^a
Balat_1097	CCTCTTCGCGGTGCTGAT/GGGATTGAACGCGAACGAT	GlcU; putative Glc uptake permease	0.92 ± 0.11
Balat_1154	GGTATTACGGCCGACGAGTT/GCCGTGCCATCGAAACC	Sugar transporter; permeases of the major facilitator superfamily	0.53 ± 0.02
Balat_0054	TTGCGCGTCATCAATGCAT/GAGTGTGGTCCGACAAATTGC	Cation symporter family protein	0.82 ± 0.08
Balat_0475	GCGCGCGACATCTCCTATT/AAGTGTAGACGCTGCGTTCATG	LacS; galactoside symporter	23.95 ± 1.1
Balat_0483	CAGCACACGCGCAATCG/CCACAGGCTGCCAATGGTA	MalE-type ABC sugar transport system periplasmic component	0.39 ± 0.16
Balat_1240	TGTTCCACGCCATTGAAGTTC/ACTTCACGCTCCACTTCAATCC	LacY; galactoside permease	0.28 ± 0.02

^a The expression of the genes in cells grown in Lac relative to that in cells grown in Glc is represented.

side symporter], Balat_0483 [MalE-type ABC sugar transport system periplasmic component], and Balat_1240 LacY, galactoside permease]). Only one of these genes, Balat_0475, was found to be up-regulated in cells grown in the presence of Lac (Table 2).

DISCUSSION

The increased interest in the utilization of *B. animalis* subsp. *lactis* strains to promote beneficial effects on human health, as well as the study of the metabolism of different carbon sources, has drawn considerable attention to this species. The metabolism of bifidobacteria may play a role in the beneficial effects attributed to these microorganisms. Furthermore, lactose utilization is of key importance to guarantee the viability of this bacterium in functional dairy products. Therefore, knowledge of sugar metabolism and transport in bifidobacteria is necessary to understand the activity of bifidobacteria in both the gut and fermented foods. However, the heterofermentative metabolism of bifidobacteria has been poorly characterized so far. In this work, ¹³C NMR was used to trace the fate of labeled carbons of Glc and Lac throughout metabolic pathways. To date, *in vivo* metabolic studies, at the level of metabolites, have not been performed in bifidobacteria.

Several studies have shown differences between *Bifidobacterium* species growth and the carbon sources used. Our *in vivo* ¹³C NMR results showed that Glc consumption was faster than Lac consumption. Furthermore, the time of consumption of galactose moieties of Lac was higher than the time of consumption of Glc moieties of Lac.

Theoretically, 1.5 mol of acetic acid and 1.0 mol of lactic acid are formed from 1 mol of Glc through the bifid shunt (26). Changes in the direction of the pathway toward the formation of one or the other end product could modify the final energetic yield and redox balance. The displacement toward acetic acid formation in the bifid shunt allows extra ATP generation, whereas the displacement toward lactic acid formation allows the production of extra NAD⁺ yield and, therefore, a greater capability to equilibrate the redox balance of the cell (33). The higher acetic acid/lactic acid ratios compared with the theoretical ratio observed under our experimental conditions indicate that the bifid shunt is displaced toward acetic acid formation. Remarkably, the acetic acid/lactic acid ratio from Glc metabolism was higher than the ratio from Lac metabolism. In the bifid shunt, acetyl phosphate, either from xylulose 5-phosphate or from fructose 6-phosphate, is converted to acetate by AckA, with the production of 1 mol of ATP per mol of acetic acid. The activity of AckA was higher in cells grown in the presence of Glc, indicating that under these conditions the bifid shunt is displaced toward the production of acetate.

The higher concentration of acetic acid found in these experiments also suggests the formation of larger amounts of ATP in the cell. Ldh activity was found to be increased in cells grown in the presence of Lac, indicating that, under those conditions, the bifid shunt is displaced toward the production of lactate. This is in agreement with previous studies showing that in the presence of certain fermentable sugars (maltose), *B. animalis* subsp. *lactis* has a greater ability to balance the redox status of the cells (33). The differences in the activities of the enzymes tested (AckA, Ldh) were in agreement with changes in the levels of metabolic end products (acetate and lactate) and showed that the two bifid shunt branches are differentially activated depending on the carbon source. Remarkably, other authors have shown that the ratios of lactate, formate, and acetate were altered between and within *Bifidobacterium* species growing on different carbohydrates (34).

Sugar transporters often constitute the bottleneck of sugar metabolic pathways, controlling the carbon flux rate. Our results suggest that the BB-12 β-galactosidase(s) and its putative Lac transporter, Balat_0475, work quickly to introduce and degrade the Lac. However, the galactose moieties of Lac were metabolized far more slowly. Similar results have been reported in *Streptococcus thermophilus*, in which Lac is taken up by a galactose/Lac antiporter, LacS, and immediately hydrolyzed and the galactose moieties of Lac are slowly metabolized (35). Regarding Glc transport, one study has recently suggested that Glc is introduced into the cell by facilitated diffusion in *B. animalis* subsp. *lactis* DSMZ 10140 (36). Also, Briczinski and coworkers (37) correlated the inability of some strains of *B. animalis* subsp. *lactis* to transport Glc and to grow rapidly in a medium containing Glc as the sole carbon source with the presence of nonsynonymous single-nucleotide polymorphisms within the *glcU* gene.

Our qRT-PCR results suggest that *lacS* (Balat_0475) is implicated in Lac transport in BB-12. The genome sequences of *B. bifidum* PRL2010 and *B. longum* NCC2705 revealed the presence of *lacS* homologs on both genomes (38, 39). In fact, in *B. longum* NCC2705, *lacS* was found to be upregulated during growth in human milk (40).

An interesting observation of this study regards Pfl activity. Pfl is the enzyme that catalyzes the conversion of pyruvate and CoA to formate and acetyl-CoA. So far, this reaction was assumed to be unidirectional toward the formation of formic acid, but our experiments suggest that Pfl can catalyze a bidirectional reaction in *B. animalis* subsp. *lactis* under certain physiological conditions. Acetate should be the exclusive labeled end product derived from the metabolism of both Glc and Lac labeled on carbon 1 of the Glc moiety and processed via the bifid shunt. This labeling pattern

results from the combination of the asymmetric fructose 6-phosphate phosphoketolase split of fructose 6-phosphate and xylulose 5-phosphate and the pentose phosphate pathway (PPP) activities of transaldolase and transketolase (Fig. 6). However, labeled lactate on carbon 1 was consistently detected throughout our experiments. The label on C-1 of lactate could arise from an active Embden-Meyerhof-Parnas (EMP) pathway or from a Pfl with bi-directional activity which could catalyze the condensation of formate and labeled acetyl-CoA resulting from Pta activity (Fig. 6). To test the latter hypothesis, we determined the Pfl activity pushing the reaction toward the formation of pyruvate under saturating conditions of substrate. Our results confirmed the ability of Pfl to catalyze the conversion of formate to pyruvate in the presence of acetyl-CoA. In addition, a gene encoding the key enzyme of the EMP pathway, the 6-phosphofructokinase, is not present in the genome sequence of BB-12 (41).

Furthermore, the presence of the bifid shunt in BB-12 was fully confirmed by analyzing the labeling profile of end products derived from the metabolism of sugar substrates specifically labeled in carbons of choice in perchloric acid extracts. These experiments allowed the differentiation of the bifid shunt from the other metabolic pathways: EMP and PPP. The [1-¹³C]acetate, [2-¹³C]lactate, and [1-¹³C]ethanol generated after BB-12 [2-¹³C]Glc metabolism allowed discrimination between the bifid shunt and the PPP. This was further corroborated by the labeling pattern resulting from [1-¹³C]Glc, which, when processed by the PPP led to ¹³CO₂ and, thus, a loss of labeled carbon. The [1,2-¹³C]acetate, [2,3-¹³C]lactate, and [1,2-¹³C]ethanol generated after [3-¹³C]Glc metabolism in BB-12 allowed discrimination between the bifid shunt and EMP, which led to the production of only [1-¹³C]lactate as the labeled end product from [3-¹³C]Glc. These results agree with the data proposal by Wolin and coworkers (20). In conclusion, our results demonstrate that *B. animalis* subsp. *lactis* BB-12 exclusively uses the bifid shunt for the fermentation of Glc and Lac.

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