Lactobacillus acidophilus NCFM is a probiotic bacterium adapted to survive in the gastrointestinal tract and with potential health benefits to the host. Lactitol is a synthetic sugar alcohol used as a sugar replacement in low calorie foods and selectively stimulating growth of L. acidophilus NCFM. In the present study the whole-cell extract proteome of L. acidophilus NCFM grown on glucose until late exponential phase was resolved by 2-DE (pH 3–7). A total of 275 unique proteins assigned to various physiological processes were identified from 650 spots. Differential 2-DE (DIGE) (pH 4–7) of L. acidophilus NCFM grown on glucose and lactitol, revealed 68 spots with modified relative intensity. Thirty-two unique proteins were identified in 41 of these spots changing 1.6–12.7-fold in relative abundance by adaptation of L. acidophilus NCFM to growth on lactitol. These proteins included β-galactosidase small subunit, galactokinase, galactose-1-phosphate uridylyltransferase and UDP-glucose-4-epimerase, which all are potentially involved in lactitol metabolism. This first comprehensive proteome analysis of L. acidophilus NCFM provides insights into protein abundance changes elicited by the prebiotic lactitol.