

Treatment of Lactose Intolerance via β -Galactosidase Overexpression in Supplemental Microflora

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INTRODUCTION

Lactose intolerance, or hypolactasia, is a condition affecting 65% of people worldwide, and results from a genetic inability to produce sufficient quantities of the enzyme lactase in the small intestine. During normal lactose degradation, ingested lactose from cheese, milk, etc is cleaved by lactases into two conjugate sugars, glucose and galactose, within the duodenum of the small intestine. However, when secreted lactases are inviable or insufficient in quantity to degrade the ingested lactose, then the lactose continues to the large intestine, where native microbes break the molecule down in processes that produce gaseous byproducts, including hydrogen, carbon dioxide, and methane. The buildup of these gases causes the bloating and cramps associated with lactose intolerance, and can increase the osmotic potential, causing excess water accumulation within the large intestine. (*LCT*, *Genetics Home Reference*)

In humans, lactase expression is regulated by the *LCT* gene (sometimes called *LAC*, *LPH*, or *LPH1*), a 49,341 base pair gene on position 21 of the q arm of chromosome 2. Although lactose intolerance is often viewed as affecting only a minority of the population, approximately 65% of people worldwide have a decreased ability to metabolize lactose. This percentage varies significantly across different populations, with East Asian and African populations showing as high as 90%, and Northern European populations as low as 5%. All mammals are able to metabolize lactose in infancy, as lactose is the primary sugar in milk, but this ability decreases dramatically due to downregulation of the *LCT* gene after weaning. Only a minority is said to be "lactase persistent" into adulthood, meaning that the ability to metabolize lactose is never lost (*LCT*, *Genetics Home Reference*).

Hypolactasia is inherited as an autosomal, recessive phenotype, and is lactase persistence is strongly correlated with two single-nucleotide polymorphisms (SNPs) upstream of *LCT* in the *MCM6* gene. The gene product of *MCM6* (Minichromosome Maintenance Complex Component 6) is a component of a hexameric protein complex with DNA helicase activity, and the gene includes regulatory elements for the *LCT* gene. Two SNPs in *MCM6*, C/T-13910 and to a lesser degree G/A-22018, strongly correlated with the lactase

persistence phenotype (Enattah, NS. et. al. 2002). The region of these SNPs acts as a *cis* element that can silence or enhance activation of the *LCT* promoter (Olds, LC. and Sibley, E. 2003).

The gene encodes the 1924 amino acid protein lactase, also called β -Galactosidase. Lactase is an integral plasma membrane protein expressed by epithelial cells of the small intestine. The enzyme catalyzes the hydrolysis of the β -glycosidic bond in D-lactose to form D-glucose and D-galactose. Although the exact mechanism of lactase's activity is not certain, the prevailing theory states that the Glu537 of the lactase active site acts as a nucleophile which binds covalently with the β -glycosidic carbon of the galactosyl group. The D-glucose leaving group is then assisted by either Mg^{2+} or Glu461. The D-galactose then detaches from Glu537 due to nucleophilic attack by water (Juers, DH. et. al., 2001).

There are several methods of managing lactose intolerance, including avoidance, treatment, or potential cures. Individuals with lactose intolerance generally avoid their symptoms by simply abstaining from the consumption of lactose-containing products, or by consuming special dairy products that have had the lactose previously removed; however, these are simply preventative measures. Actual treatments include lactase supplements, such as Lactaid®, as an artificial supply of lactase. One study showed that such supplements can be helpful in preventing the clinical symptoms associated with lactose intolerance: children given a lactase supplement immediately prior to ingestion of a lactose solution exhibited significantly less hydrogen (7 ppm) in their breath, and did not experience diarrhea, bloating, or abdominal pain when compared to the control group, showing hydrogen breath of 60 ppm, and a much higher occurrence of the aforementioned symptoms (Medow et. al., 1992). However, ingested supplements are often ineffective due to degradation of the lactase within the low pH environment of the stomach: researchers exploring the effectiveness of four common commercially available lactase supplements found that the enzyme activity fell dramatically to 0-65% when exposed to the full *in vitro* digestive environment (O'Connell, S. and Walsh, G., 2006). In addition to their questionable effectiveness, lactase supplements can be expensive over time and inconvenient for users, who would

need to be mindful to bring the pills and ingest them prior to any time they wished to consume lactose.

Although there are some limited treatment options for lactose intolerance, cures do not yet exist. Because hypolactasia is a genetic disorder, a cure would require gene therapy, in which a functional lactase gene could be delivered and incorporated into the host's genome. Attempts at gene therapy have used adenoviruses and adeno-associated virus (AAV), which would behave as non-pathogenic viral vectors to deliver a corrected copy of a desired gene to the recipient. Unfortunately, this approach is currently infeasible due to the limitations in gene therapy technology. Integration of a corrected gene is not precise, so the corrected gene can disrupt the sequence of some other important gene, creating new problems. The problem of insertion inaccuracy is compounded by the multicellularity of a human host: the gene must not only be inserted accurately, but it must be inserted accurately in trillions of target cells. While recent experiments have had increasing success with accurate gene insertion, the immune system presents additional problems. Although AAV is not known to be pathogenic, it can be mildly immunogenic, causing rejection of the AAV by the innate immune system. B cell mediated humoral immunity eliminates extracellular AAV while cytotoxic T cells eliminate host cells that have received the corrected gene from the virus (Daya, S. and Berns, K., 2008). Gene therapy is very promising, but must overcome many limitations before it could be used as a viable cure for lactose intolerance. However, the idea that a functional lactase gene could be delivered to a cell that would then produce the enzyme can be applied to a far more manageable, well-characterized host.

The human body hosts a diverse microflora consisting of nearly ten times more bacterial cell than human cells (Savage 1977). The majority of the bacteria live symbiotically within the host's digestive tract. These bacteria, being unicellular organisms, opposed to a multicellular organism with several trillion cells, can be much more easily transformed with exogenous DNA. Due to their relative ease in transformation, and ubiquitous presence in the digestive tract, bacteria could be manipulated for the purpose of lactose intolerance treatment or cure. In this project, a non-pathogenic bacterial species, *Escherichia coli* was transformed with exogenous DNA for the enzyme β -galactosidase (β -gal). Prior to transformation, the β -gal gene was inserted into a plasmid with an overexpression promoter sequence so that the transformed bacterial cell would produce copious quantities of β -gal. If a population of this *E. coli* strain were incorporated into the digestive

microflora of a lactose-intolerant host, then ingested lactose would be correctly degraded by the transformed bacteria within the small intestine before the lactose could reach the problematic bacteria within the large intestine. If this method is found to be an effective means of degrading lactose, then lactose intolerant individuals could theoretically take these bacteria as a probiotic supplement, and establish a permanent internal microflora that could hydrolyze ingested lactose for the individual. This probiotic supplementation would be superior to previous treatments for lactose intolerance because the individuals would be able to freely consume and enjoy lactose-containing products without fear of symptoms. The probiotic's permanent habitation of the gut means that the individuals would never need to abstain from lactose, nor would they need to constantly purchase, carry around, and consume a β -galactosidase supplement any time they wished to consume lactose—the β -galactosidase would already be present and ready in the gut, just as it would for those without lactose intolerance. This one-time treatment would require ingestion of only a few probiotic pills until the strain was established in the gut, where the probiotic would propagate and persist mutualistically with the host.

Principal Approaches & Methods

Bacterial Host Selection

To determine the ideal host for transformation with the overexpression plasmid, and to function as the final probiotic, potential bacterial candidates were chosen based on their natural prevalence in the human gut, and for their propensity for pathogenicity. Three genera, *Bacteroides*, *Lactobacillus*, and *Bifidobacterium*, were originally considered, but were eliminated for different reasons. *Bacteroides* constitutes a large percentage of the gut microflora, but can be an opportunistic pathogen. *Lactobacillus* is a common probiotic found in yogurt, but cannot survive well in the gut. *Bifidobacterium* is another common probiotic, but is an obligate anaerobe, making it impractical for work in a lab setting. Eventually, *Escherichia coli* was chosen because it is extremely well-characterized, nonpathogenic (aside from strain O157:H7), native to the human gut microflora, and is simple to culture. Specifically, DH5 α *E. coli* was used due to its facilitative use for transformation and recombinant DNA cloning methods.

Plasmid Selection

To serve as a plasmid vector that would constitutively overexpress the DNA insert, *β gal*, the

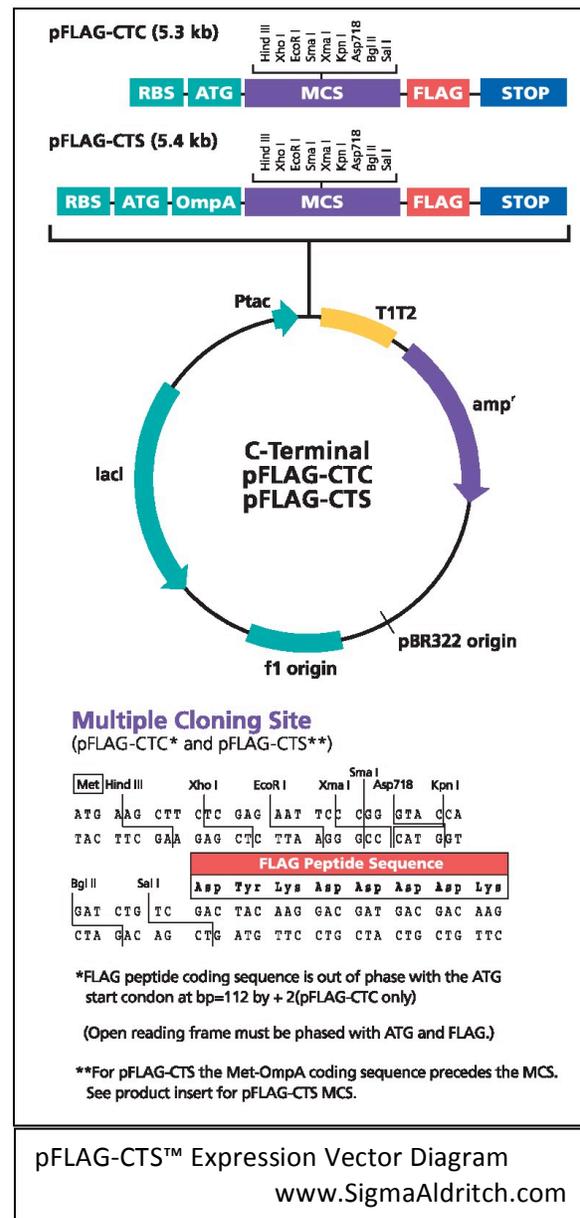
Sigma-Aldrich pFLAG-CTS™ Expression Vector and pFLAG-CTS™-BAP Control Plasmid were selected. Specially made for use in *E. coli*, the expression vector was a 5403 bp plasmid with a *tac* promoter that would constitutively express the insert and secrete the product into the periplasmic space. The sequence included a multiple cloning site and an 8 amino acid C-terminal FLAG® for purification of the product. For purposes of cell selection, the plasmid included an ampicillin resistance gene (*Beta-lactamase*). The control plasmid, 6735 bp, is functionally similar to the expression vector, except that it is used for periplasmic expression of FLAG®-BAP, for comparative use in ELISA, Western Blotting, etc.

Bioinformatics and Primer Design

Two genomic sources were chosen from which to clone the β -gal gene, *E. coli* and *Aspergillus fumigatus*: cloning of the human *LCT* gene was not attempted because its large size—49+ kb—would prevent its insertion into a plasmid. *A. fumigatus* β gal was chosen as a candidate because of its use in commercially available β -gal pills, and *E. coli* β gal (also called *LacZ*) was chosen for its workable size (3072 bp) and efficiency in the *E. coli* host. BLAST search was used to compare an *A. fumigatus* DNA sequence to human *LCT* and confirm that the predicted *A. fumigatus* β -gal gene was indeed an ortholog to human *LCT*, and that both sequences had upstream HindIII and downstream BglII restriction sites for insertion into the expression vector's multiple cloning site. Once the *A. fumigatus* and *E. coli* β -gal sequences were acquired, four 33-34 bp primers were designed and ordered from Integrated DNA Technologies (IDT®).

Genomic DNA Extraction

A. fumigatus genome extraction was unnecessary because a cDNA genome was already available in the lab. To extract the DH5 α *E. coli* genomic DNA, a phenol/chloroform method was used. An overnight culture of *E. coli* was pelleted and resuspended in 600 μ L Lysis Buffer (9.34 mL TE buffer, 600 μ L 10% SDS, and 60 μ L proteinase K (20 mg/mL)), then incubated 1 H at 37 degrees C. After addition of 600 μ L 1:1 phenol/chloroform and mixing, the suspension was centrifuged at max speed and the aqueous layer was pipetted away then gently mixed with -20°C 100% EtOH. After a 30-minute incubation at -20°C, the suspension was centrifuged at max speed for 15 min at 4°C. After centrifugation, the supernatant was discarded, and the pellet was resuspended in 1 mL of room temperature 70% EtOH. After a final centrifugation, the supernatant was discarded, the pellet was allowed to dry, and then was resuspended in 100 μ L TE buffer (10 mM Tris-Cl (pH 8), 1 mM EDTA (pH 8)).



Cloning and Extraction of *B-galactosidase*

Cloning of β -gal was accomplished via PCR (Polymerase Chain Reaction) amplification, using PCR cocktail concentrations described by Phusion® High-Fidelity PCR. Thermal cyclor settings were set to: 3 min 95° C hot start, and 39 cycles of 1 min 95° C denaturation, 1 min 52° C annealing, and 4 min 72° C elongation.

For extraction of amplified β -gal, the gel electrophoresis in 1% agarose was used to isolate bands. β -gal gel bands were excised, weighed on an analytical balance, and purified as per the QIAGEN QIAquick Gel Extraction Kit protocol.

Insert and Vector Preparation

The β -gal insert and the expression vector were each treated to restriction digests with appropriate concentrations of NE Buffer III, HindIII, and BglIII. The digests were incubated at 37°C for 12 H, and then purified via QIAGEN QIAquick PCR Purification Kit and protocol.

To prevent the vector from self-ligating again, the vector was then treated with CIP Alkaline Phosphatase for 90 min at 37°C. After treatment, the vector was purified with the QIAquick PCR Purification Kit.

Vector and Insert Ligation

To achieve a 5:1 insert:vector ratio for the ligations, 57.37 ng insert were added for every 20 ng vector. The ligation was prepared with the appropriate concentrations of T4 Ligase, Ligase Buffer, and insert:vector. In addition to the ligations, a vector only (no β -gal insert added) control was prepared. The ligations and vector only sample were then incubated overnight at 16°C.

Transformation

Chemically competent DH5 α *E. coli* cells were prepared via the *Mix & Go E. coli* Transformation Kit and protocol, and stored at -80°C. When ready to be transformed, 50 μ L aliquots of prepared cells were thawed and gently mixed with 5 μ L of plasmid from either the ligation or the vector only sample. The transformants were then plated on prewarmed (37°C) LB-Amp plates, and incubated overnight at 37°C.

Transformant Screening

Colonies were selected at random from the ligation plates, and each grown overnight in LB Amp broth in a 37°C shaker. Plasmid DNA was then purified using a QIAGEN QIAprep Spin Miniprep Kit and protocol. Each purified plasmid sample was then treated to a restriction digest with NE Buffer III, BglIII, and HindIII for 2 H at 37°C. The digests were then run on a 1.5% agarose gel to determine if the sample showed two bands: a 5403 bp band representing the expression vector and a 3072 bp band representing the β -gal insert.

Results

Cloning and Extraction of β -gal

Initial attempts to clone β -gal from genomic *E. coli* DNA and *A. fumigatus* cDNA were unsuccessful when using traditional *taq* polymerase. Gel electrophoresis *should* have shown a single band at 3100 bp (representing the amplified β -gal gene, but results (Fig. 1) showed bands appearing around 1400 bp and 1200 bp in *E. coli* sample wells B and C, and other bands appearing around 150 bp and 250 bp in the *A. fumigatus* sample well, D. However, a faint band at 3100 bp can be seen in well C, showing some replication of the β gal gene.

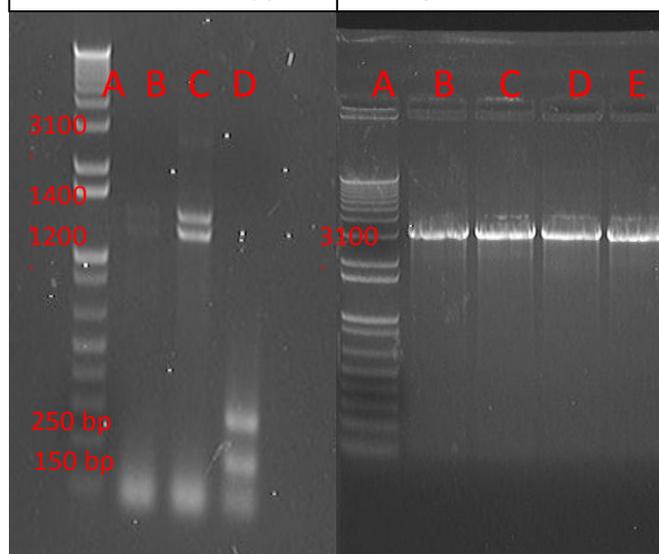
Later attempts to clone β gal with Phusion® High-Fidelity *taq* polymerase proved more successful, as shown by the gel electrophoresis results (Fig. 2). All four samples (wells B-E) show well defined bands in the position of around 3100 bp, the expected band size for β gal.

Transformation

As expected, transformation of the DH5 α *E. coli* cells with the ligations (the expression vector and β gal insert) resulted in colony growth for all three replicates, plates A-C, as recipients of the vector would also acquire the Amp^R (β -lactamase) gene, allowing them to survive on plates with ampicillin.

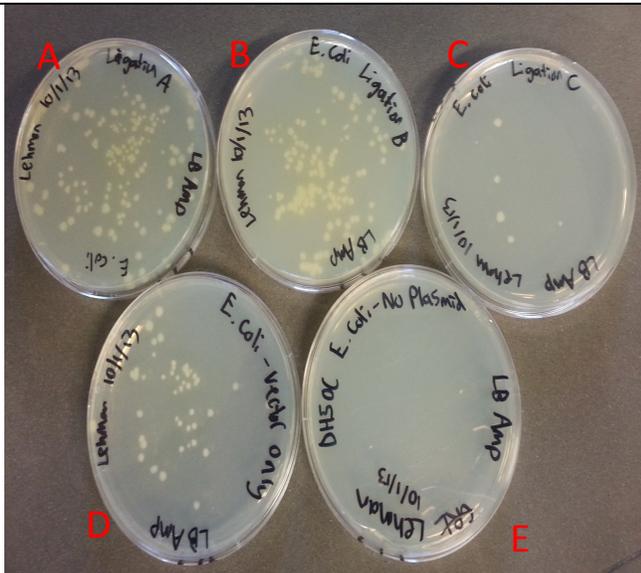
Figure 1. Agarose gel depicts failure of traditional *taq* polymerase to clone β gal specifically during PCR, as no bands appear

Figure 2. Agarose gel depicts success of Phusion® High-Fidelity *taq* polymerase to clone β gal with high specificity during PCR. **A)** 1 KB Plus



Growth of the colonies shows that transformation of the cells was successful. However, there was also unexpected colony growth for cells transformed with only the expression vector cleaved by CIP alkaline phosphatase. Cells should not have grown, as the

Figure 3. Depicts the plated transformants on LB Amp after 24 H at 37° C. **Plates A-C** are replicates of the ligations of expression vector and the *βgal* insert. Growing colonies indicate that transformation was successful. **Plate D** depicts cells transformed with only the vector (no *βgal* insert)



vector, theoretically, could not re-ligate due to its removed 5' phosphate group, preventing expression of the Amp^R gene.

Screening of Ligation Colonies

Screening of candidates from the ligations was problematic due to inconsistent gel results. Through multiple gel attempts, isolated plasmid DNA did not show the predicted band pattern—a 5403 bp band and a 3072 bp band. Instead, the wells showed a solid streaking pattern in the wells, a sign of DNA degradation. Screening was retried with colonies from new ligations, and each sample was run on the gel with and without the restriction digest step. No differences in banding pattern were observed between a given restricted sample and its respective unrestricted sample. However, each different ligation colony showed multiple random bands, nowhere in the expected range.

Conclusion

The difficulty in verifying the successful creation of the final plasmid construct (expression vector+*βgal* insert) could be the result of a multitude of factors. It was originally predicted that the streaking pattern of the ligation candidates' plasmid DNA could be the result of the restriction enzymes, HindIII and BglII, spoiling. The streaking bands signify DNA degradation, and if the restriction enzymes had been contaminated or otherwise compromised, it could result in the enzymes' nonspecifically cleaving the plasmid DNA at multiple loci, instead of only the desired restriction sites. However, when new sets of candidate plasmid DNA—one set unrestricted and the other treated to the restriction digest—were run on a gel, there was no difference in banding pattern between a restricted sample and the respective unrestricted sample. The absence of any different between the samples shows that the restriction enzymes were not the source of the error. Instead, the length of the gene of interest could have been a contributing difficulty: *E. coli β-gal*, at 3072 bp, is much longer than usual cloning targets. The gene length was proven to be an obstacle when traditional *taq* polymerase was insufficient to replicate the gene with high-fidelity. Instead, the Phusion[®] High-Fidelity *taq* polymerase had to be used to amplify the target gene from the *E. coli* genomic DNA.

Although the fidelity issue was surpassed with the Phusion[®] *taq*, the gene length could have been a continuous source of difficulty, as low DNA yield was a continuous problem throughout the project. DNA quantification and purity measurement via A260:A280 nm fluorescent plate reader consistently showed significant DNA quantity loss after each purification step. The length of *β-gal* could have been a contributing factor to reduced yield, or the perhaps the loss was due simply to limitations in current purification technology.

After prolonged trial-and-error with the original expression vector and DNA insert prepared for ligation (which yielded the ligation candidates that produced random or streaking gel bands), new expression vector and *β-gal* DNA were prepared from the DH5α *E. coli* genomic DNA. During preparation of the new vector and insert, it was determined that there had been a mistake during the alkaline phosphatase treatment of the original vector and insert set. The *β-gal* insert had mistakenly been given the alkaline phosphatase treatment instead of the intended target, the expression vector. This mistake with the alkaline phosphatase treatment was likely the largest contributing factor to the later difficulties in screening. This error meant that the expression vector could self-ligate and recircularize, even after the restriction digest, thus reducing the number of potential ligation targets for the *βgal* insert. Additionally, the *βgal* insert would have had its 5' phosphate removed, reducing the insert's binding

affinity for the expression vector's multiple cloning site. These compounding factors would result in a dramatically reduced quantity of successfully formed βgal expression vectors after the ligation step, and is most likely the cause of difficulty during the screening process.

After the alkaline phosphatase error was discovered, brand new βgal insert and expression vector were prepared. Transformation of DH5 α *E. coli* with the correctly prepared and ligated βgal overexpression plasmid has yielded promising new candidates for screening. If it is verified that these new candidates contain the desired βgal overexpression construct, then the new *E. coli* strain can be examined for the next stage of testing.

Future Direction

Once the new *E. coli* strain is verified to contain the desired construct, the degree of βgal expression can be determined and compared against DH5 α *E. coli* without the plasmid. An extraction of the strain's mRNA, followed by Reverse Transcription-PCR (RT-PCR) of the βgal mRNA would yield a quantity of cDNA βgal copies directly proportional to the number of βgal mRNA being expressed. From the βgal cDNA, quantitative PCR (qPCR) could be used to amplify the cDNA and determine the degree of βgal expression in the new strain versus a control strain. To determine that the mRNA βgal transcripts were successfully translated to protein, a sandwich ELISA (Enzyme-Linked Immunosorbent Assay) could be used. Because the pFLAG-CTS™ Expression Vector features a C-terminal FLAG®, commercially available anti-FLAG antibodies could be used to extract the βgal protein expressed by the strain. Addition of a chromogenic substrate, such as horseradish peroxidase, would produce a color change proportional to the quantity of βgal protein in the sample.

Finally, a simple x-gal plate could be used to determine if the secreted βgal retained its functionality. If the βgal is still functional, then the growing colony would be blue due to the blue byproduct of x-gal's hydrolysis via βgal . If function was lost, then the colonies would appear white. This method could potentially be expanded to liquid x-gal culture. Because the enzymatic hydrolysis produces a blue byproduct, a spectrophotometer could be incorporated to measure color change in the *E. coli* culture over time. Such a method would allow measurement of the new *E. coli* strain's βgal enzyme kinetics versus that of a control strain.

If shown to express high quantities of βgal , this transformed *E. coli* strain could be tested as a potential probiotic supplement to treat lactose intolerance in mice trials. If mice given the probiotic exhibited alleviated lactose intolerance symptoms

versus a control group, then it would support the idea of using transformed probiotics as a viable means of lactose intolerance treatment. This method could potentially be used to help some of the billions of people worldwide who suffer from a lactase deficiency, and would support future research in the concept of treatment via recombinant probiotics.

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