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In-silico mutagenesis of L- asparaginase enzyme derived from three microbiome strains: Potential implications for cancer treatment

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ABSTRACT

With possible ramifications for cancer treatment, this work examined how silico mutagenesis affected the L-asparaginase L-(ASNase) enzymes from three microbial sources: Bifidobacterium longum, Lactococcus lactis subsp. lactis, and Escherichia coli K12. The influence of mutagenesis on the binding affinity of L-asparagine was assessed by substituting a single amino acid at predicted active site residues using computational techniques such as molecular docking, active site prediction, and 3D protein modeling. Four distinct mutations of L-ASNase in E. coli (GLY198CYS, GLY198LEU, LEU190LYS, and PRO194ALA) showed a marginally higher binding affinity (-5.9 kcal/mol) than the wild-type enzyme (-5.8 kcal/mol). The 290–298 region was where L. lactis mutations exhibited clustering effects, with position 295 (ASN295) exhibiting mutational sensitivity. It's interesting to note that B. longum L-ASNase showed consistent binding reduction across all studied mutations (-5.8 kcal/mol versus wild-type -5.6 kcal/mol), indicating that its active site residues have evolved to be more efficient. Enzyme-substrate interactions are influenced by changes in charge distribution, steric effects, hydrogen bonding capacities, hydrophobicity, and secondary structure integrity, according to a thorough examination of these mutations. Even though there were only slight changes in binding energy, our research offers important new information on the structure-function relationship of L-ASNase enzymes and suggests possible targets for enzyme engineering to improve therapeutic efficacy in cancer treatment applications.

Keywords: *In-silico, Mutagenesis, L-asparaginase enzyme, Microbiome, Cancer treatment, Molecular docking, Computational drug design*

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1. INTRODUCTION

A class of disorders known as cancer is defined by the body's aberrant cells growing and spreading out of control. (Karn et al., 2022). Among the most common types of cancer are leukemia, breast cancer, lung cancer, prostate cancer, colorectal cancer, and skin cancer (Ferlay et al., 2021). Depending on the type and stage of the cancer, there are different therapy options. The most widely used therapeutic approaches are hormone therapy, immunotherapy, targeted therapy, radiation therapy, chemotherapy, and stem cell transplantation (Zugazagoitia et al., 2016). Traditional methods such as chemotherapy, radiation, and surgery are still seen to be useful, but certain more efficient therapeutic approaches are required to avoid cancer because of specific adverse effects (Esfahani et al., 2020). Many cancer patients either quickly acquire resistance to immunotherapy or do not respond to treatment (Sambi et al., 2019). There is a growing need to investigate novel, more effective, and less harmful therapy approaches in order to address these issues.

Drugs based on microbial enzymes present a viable path toward the development of novel anticancer treatments, with the potential for increased effectiveness while reducing side effects (Yari et al., 2017). The primary benefit of microbial enzyme synthesis is the ability to produce large quantities of enzymes in a shorter amount of time on a cheap medium. Microbial enzymes can be used therapeutically as anti-inflammatory, fibrinolytic, and anticancer medicines (Germolec et al., 2018; Vijayaraghavan et al., 2019). Additionally, certain enzymes can be utilized to specifically target the critical amino acids that cancer cells need in order to create an environment that is unfavorable to their survival, development, and proliferation (Diwan et al., 2022). For instance, antineoplastic enzymes, such as hyaluronidase, can degrade the extracellular matrix of tumours thereby inhibiting their growth (Scodeller, 2014). Recombinant methioninase (rMETase) from *Pseudomonas putida* (Pp-MGL) has shown promising results in combinational therapies against cancer cells, where it is used with 5-flurouracil (5-FU) (Sharma et al., 2014; Hoffman et al., 2019). The L-arginase enzyme demonstrated a stronger anti-cancer effect through its impact on the caspase 7 and Bcl2, Bax, and Bax/Bcl2. It causes cell arrest in the G1/S phase (Selim et al., 2024). Amino acid deprivation is an approach that shows promise for the development of novel cancer therapies. Because of their rapid growth, cancer cells produce fewer enzymes overall, which leads to auxotrophy for a subset of amino acids and makes them a target for enzymes that deplete amino acids. Amino acid deprivation therapy requires the use of certain enzymes, such as L-asparaginase (L-ASNase), arginine deiminase, methionine, lysine oxidase, glutaminase, and phenylalanine ammonia-lyase (Dhankhar et al., 2020). Therapeutic enzymes can only suppress tumor cells by cutting off amino acids, since normal cells can make their own. L-ASNase (l-asparagine amidohydrolase, E.C. 3.5.1.1) catalyzes the hydrolysis of the amine group of L-asparagine to produce aspartic acid and ammonia. Bacterial L-ASNase was the first enzyme discovered as an anticancer enzyme in 1922 (Lubkowski et al., 2021). L-ASNase is regarded as a cytotoxic therapy for childhood acute lymphoblastic leukaemia (ALL) (Juluri et al., 2022; Díaz-Barriga et al., 2021). L-ASNase reduces the availability of L-asparagine and prevents cancer cells from multiplying quickly and with a prime need for exogenous asparagine (van Trimpont et al., 2022). Lymphoblasts do not express asparagine synthetase; hence, these cells must take extracellular asparagine to survive (van Trimpont et al., 2022).

L- ASNase is also recognized as one of the important food processing agents, used in the deamidation of food preparations containing l-asparagine and carbohydrates, which are cooked at temperatures above 120 °C, such as bread and other cereal products, as well as fried products based on potatoes, generating polyacrylamide (Arindam et al., 2022). These enzymes have been identified in different microorganisms, including bacteria, archaea, and eukarya (Woese et al., 1990). The most significant bacterial sources of l-ASNase are *Escherichia coli* and *Erwinia chrysanthemi* due to their high yield, stability, and effectiveness (Ashok et al., 2019). L-ASNase mechanism involves the enzymatic hydrolysis of asparagine into aspartic acid and ammonia, both of which are non-reactive, first substrate recognition and binding. L-ASNase specifically binds to L-asparagine at its active site, forming an enzyme-substrate complex. The second step is the hydrolysis reaction in which the enzyme catalyzes the cleavage of the amide bond in asparagine, resulting in the formation of aspartic acid and ammonia (Jia et al., 2021). Asparagine is a non-essential amino acid playing an important role in protein and glycoprotein biosynthesis, regulation of gene expression, and the functioning of immune and nervous systems (Wu, 2013). Bioavailability of asparagine has been shown to promote the metastatic potential of tumor cells by affecting the epithelial to mesenchymal transition (Knott et al., 2018). Thus, depletion of asparagine offers a potential mechanism for the abrogation of asparagine auxotrophic tumor cells. L-ASNase depletes the levels of plasmatic asparagine, leading to starvation and apoptosis of tumor cells (Fernandes et al., 2017). L-ASNase depletes tumor cells of asparagine, resulting in inhibition of its downstream targets like protein serine threonine kinase (p70s6k) and 4E-binding protein-1 (4E-BP1), which further suppress translation, leading to apoptosis of tumor cells (Dhankhar et al., 2020).

The L-ASNases isolated from *E. coli* and *E. chrysanthemi* have been used extensively in medicine, but growing complications like hypersensitivity, antigenicity, short half-life, temporary blood clearance, and unfavorable L-glutaminase-dependent neurotoxicity require ongoing research to find more suitable alternatives (Nguyen et al., 2018; Radadiya et al., 2020; Sobat et al., 2020). Problems with commercial l-ASNases have prompted research into better sources of the enzyme.

The chemical modification of enzymes offers alternatives to improve their therapeutic properties, such as PEG (Arslan et al., 2021). PEG-aspargase (Oncaspar) is a PEGylated form of native *E. coli*-derived L-ASNase. Compared to non-PEGylated L-ASNase, PEG-aspargase presents prolonged circulation times caused by the reduced clearance of the enzyme, leading to less frequent administrations. Additionally, PEG modifications show a reduction in the immunogenicity of the enzyme, resulting in a better tolerability profile (Heo et al., 2019). Although the enzyme has been pegylated to reduce its immunogenicity, the PEG moieties reduce the overall efficacy of the enzyme. Further, it has been observed that the drug clearance and toxicity are enhanced due to the production of anti-PEG antibodies while using pegylated forms of therapeutic enzymes ASNase and uricase (Armstrong et al. 2007; Sherman et al. 2008). Hence, the development of a suitable alternative ASNase with high activity and low immunogenicity is the need of the hour.

Computational modelling plays a crucial role in anticancer drug discovery, including the identification and development of anticancer compounds (Ma et al., 2011; Cavalcanti et al., 2019; Iqbal et al., 2021). Computational modelling can significantly accelerate the drug discovery process, making it more cost-effective and efficient (Tutone et al., 2021). Drug discovery methods such as molecular docking, pharmacophore modeling and mapping, de novo design, molecular similarity calculations, and sequence-based virtual screening have undergone significant refinement in recent decades. Bioinformatics databases, drug screening, structural design, and immunogenicity prediction offer an opportunity to find anticancer medications that are less expensive and time-consuming (Baral et al., 2020; Li et al., 2020). According to research by Baral et al. (2020), ASNaseB genes from *Streptomyces griseus*, *S. venezuelae*, and *S. collinus* have higher binding energies than those from *E. coli* and *E. chrysanthemi* and were predicted to have the lowest Kms. As-Suhbani and Bhosale (2020) demonstrated that the interactions between the ligand L-asparagine and Fusarium solani CLR-36 L-ASNase occurred at the active site with lower binding energy. Abdelrazek et al. (2019) identified possible antigenic areas of the L-ASNase sequence of *Bacillus licheniformis* and compared them to those of *E. coli* and *E. chrysanthemi*. For the L-ASNases of *E. coli*, *E. chrysanthemi*, and *B. licheniformis*, 18, 16, and 17 antigenic areas, respectively, were discovered. Belén et al. (2021) also predicted the immunogenicity of L-ASNases from nine filamentous fungi and compared the outcomes with those of *E. coli* L-ASNase, using a bioinformatics-based methodology. In silico analysis of arginine deiminase (ADI) from *P. furukawaii* indicated *P. furukawaii* ADI (PfADI) to be closely related to experimentally characterized ADIs of *Pseudomonas* sp. with proven anticancer activity. Immunoinformatics analysis indicated lower PfADI immunogenicity than MhADI (M. hominis ADI). In silico and in vitro studies establish PfADI as a potential anticancer drug candidate with improved efficacy and low immunogenicity (Dhankhar et al., 2022). By using cutting-edge computational approaches to examine the impacts of L-ASNase mutagenesis from various microbiomes on L-asparagine binding affinity, the study seeks to increase the anticancer efficacy of ASNase enzyme.

2. MATERIALS AND METHODS

3D Protein modeling and active site prediction

The amino acid sequence of ASNase from *Escherichia coli* (STRAIN K12), uniprot ID: P0A962, *Lactococcus lactis* subsp. Lactis uniprot ID: (A0A2Z3KCZ0) and BIFIDOBACTERIUM LONGUM uniprot ID: (A0A7U4H4Y4), were retrieved from the uniport database (downloaded the alfa fold of the protein, the wild type, the PDB format). The active site of the modeled protein was predicted using the CB-Dock2(1) web server

In silico mutagenesis

The predicted active site residues were subjected to in-silico mutagenesis using BIOVIA Discovery Studio 2020. Single amino acid substitutions were introduced to generate mutant protein structures, where each site was replaced with the remaining 19 amino acids and saved in PDB file format.

Ligand preparation

The 3D structures of the ligand, Asparagine (PubChem ID: 6267), were retrieved from the PubChem database in SDF format. The ligand structures were prepared using Avogadro 1.2.0 software (2), and energy minimization was performed using the MMFF94 force field.

Protein preparation and molecular docking

All proteins were prepared using Autodock tools (3). The wild-type and mutant protein structures were prepared for docking using qvina2 (4) software. This step involved preparing your inputs, receptor structures: each mutant of the amino acid substitutions introduced to generate mutant protein structures in PDB format, and a ligand structure: asparagine in PDB format (L. ASNase).

Visualization and analysis

Visualization carried on using BIOVIA Discovery Studio 2020 software (5).

3. RESULTS

Binding affinity and mutation position

Binding affinity according to positional changes, though modest, highlights potential avenues for enzyme engineering to enhance L-ASNase efficiency for therapeutic and industrial applications. Notably, the PRO194ALA mutation, by replacing a conformationally restrictive proline with a more flexible alanine, might offer a promising strategy for optimizing enzyme dynamics. Such flexibility could facilitate improved catalytic turnover or substrate accessibility, making this variant a potential candidate for further experimental validation (Fig.1).

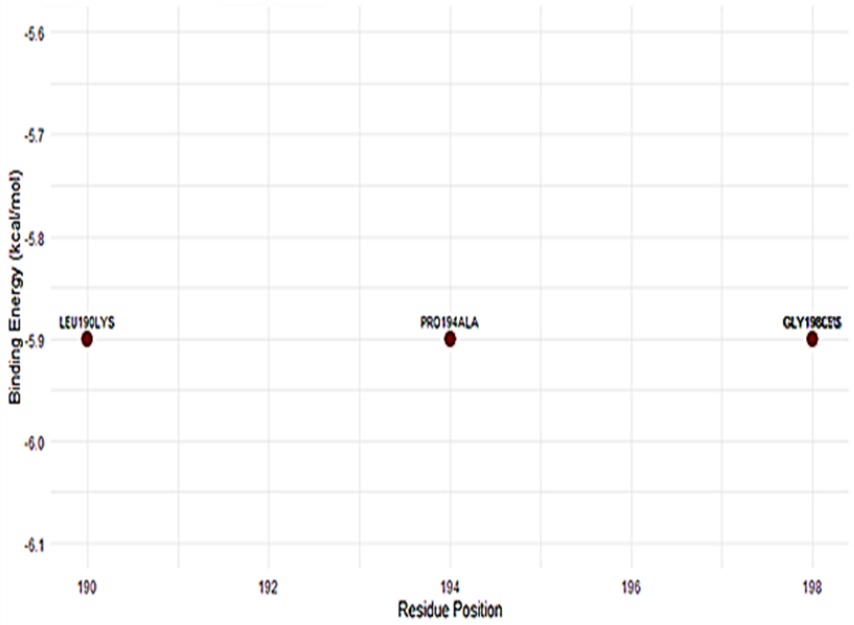

Fig. 1: Mutation position according to binding affinity

Active site analysis and mutation clustering

Mutations based on their biochemical property changes, with position shown by color and binding energy by point size. Mutations cluster into distinct groups based on their hydrophobicity and charge changes; some positions (shown by color) tend to cluster together, suggesting regional preferences for certain types of changes. The uniform point size reflects the consistent binding energy. There's a diverse range of property changes despite similar mutations based on their biochemical property changes, with position shown by color and binding energy by point size. Mutations cluster into distinct groups based on their hydrophobicity and charge changes; some positions (shown by color, Fig 2) tend to cluster together, suggesting regional preferences for certain types of changes.

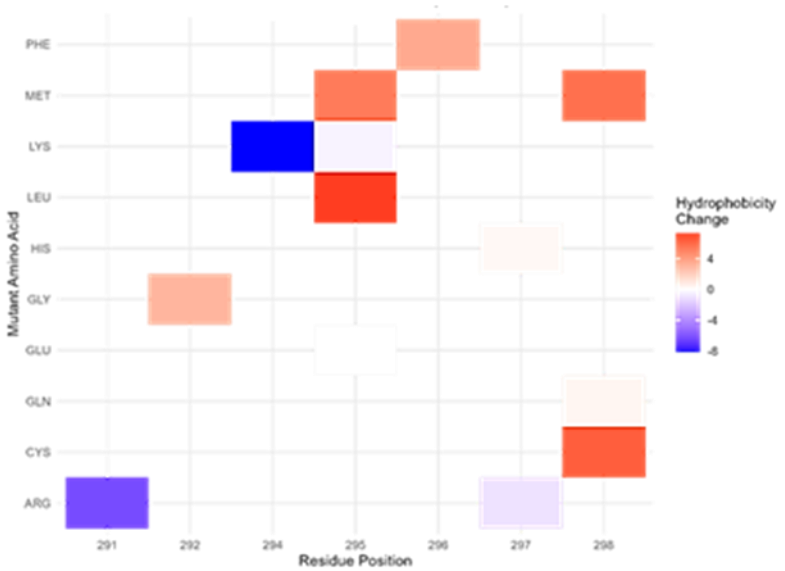

Fig. 2: Active site analysis of *L. lactis subsp*. L-ASNase.

The uniform point size reflects the consistent binding energy, and there is a diverse range of property changes despite similar binding effects. This suggests mechanisms by which different mutations achieve similar binding results (Fig. 3).

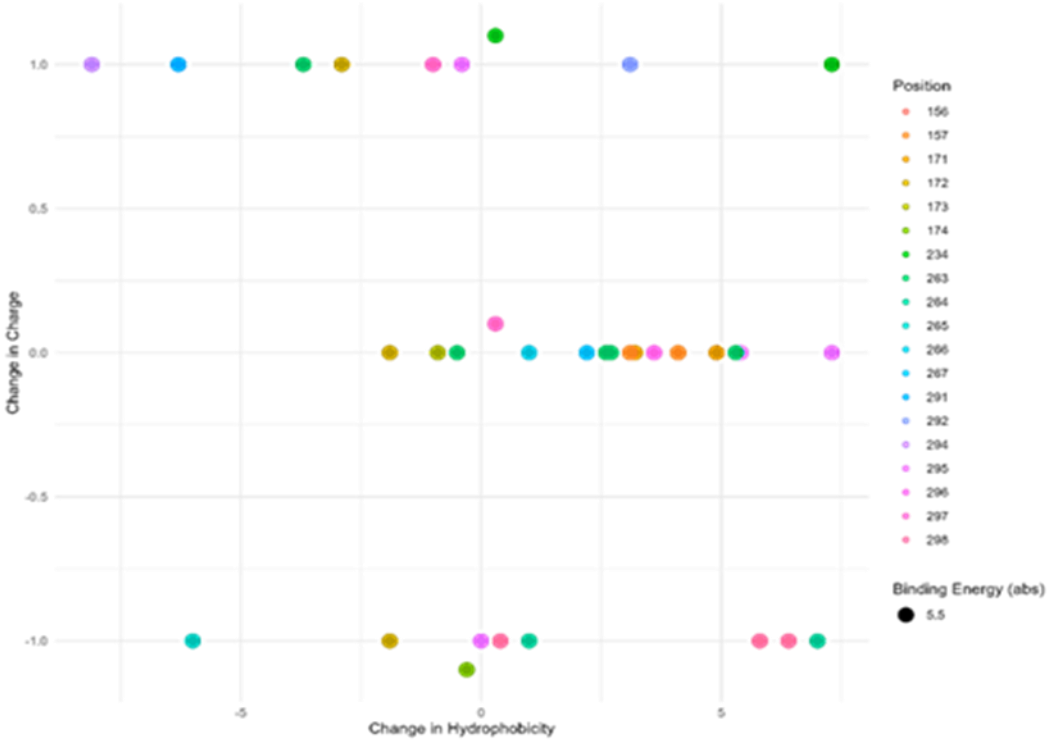

Fig. 3: Mutation clustering in *L. lactis subsp*. L-ASNase.

Mutation frequency

Fig. 4 bar chart illustrates which positions in the L-ASNase protein from *Lactococcus lactis* are most frequently mutated in the dataset. Where, Position 295 (ASN295) appears to have the most mutations, suggesting this residue may be significant for enzyme function or stability and positions 263 (SER263), 172 (PRO172), and 298 (LYS298) also have multiple mutations. However, the clustering of mutations in the 290-298 region suggests this may be part of the active site or a functionally important domain. The wide distribution of mutations across positions (157-298) indicates several protein regions may influence binding or activity.

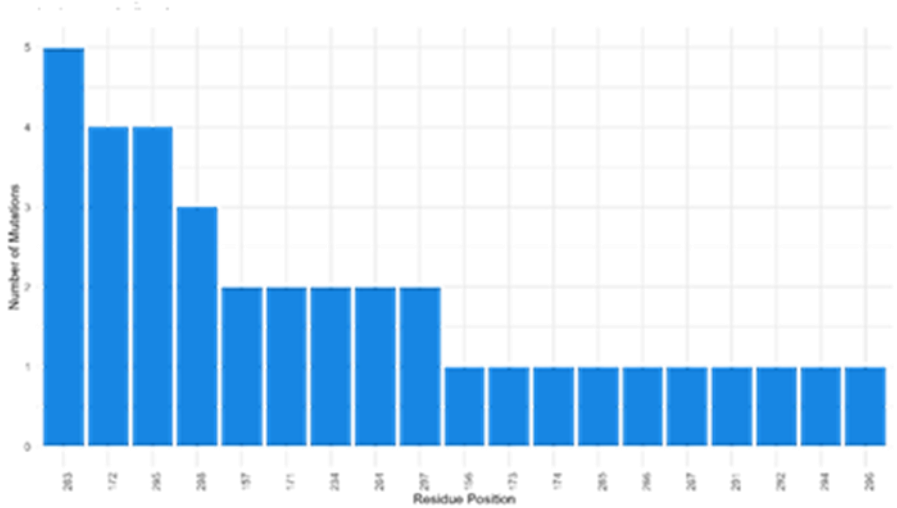

Fig. 4: Mutation frequency by position in *L. lactis subsp.* L-ASNase.

Impact of specific mutations on the binding activity

The heatmap shows all mutations organized by position (x-axis) and mutant amino acid type (y-axis), with binding energy represented by color. The uniform color indicates consistent binding energy across all mutations. However, the pattern of

mutations is not random - certain positions have multiple mutations while others have none. There are clusters of mutations in specific regions (especially around positions 290-298the impact of specific mutations on the binding and activity of the enzyme, focusing on key physicochemical properties such as charge distribution, steric effects, hydrogen bonding, hydrophobicity, and secondary structure integrity. The results indicate that while all analyzed mutations slightly enhance binding energy, their broader effects on catalytic efficiency, enzyme stability, and substrate specificity warrant further investigation (Fig. 5).

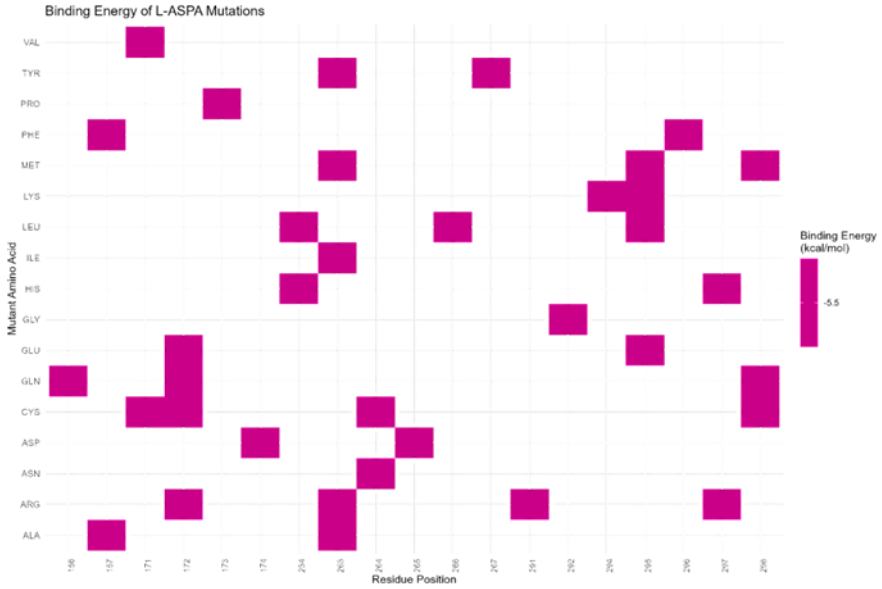

Fig. 5: Mutation heatmap *L. lactis subsp.* L-ASNase.

The analysis of amino acid substitutions in *B. longum* L-ASNase provides crucial insights into how structural modifications impact enzyme binding and activity. The binding scores for all examined mutations were found to be -5.8, which is slightly lower than the wild-type score of -5.6. This subtle but consistent decline suggests that any substitution at these specific positions negatively affects binding affinity. The uniformity of these scores implies that the enzyme exhibits low tolerance to mutations at these sites, likely due to the functional and structural importance of these residues (Fig. 6)

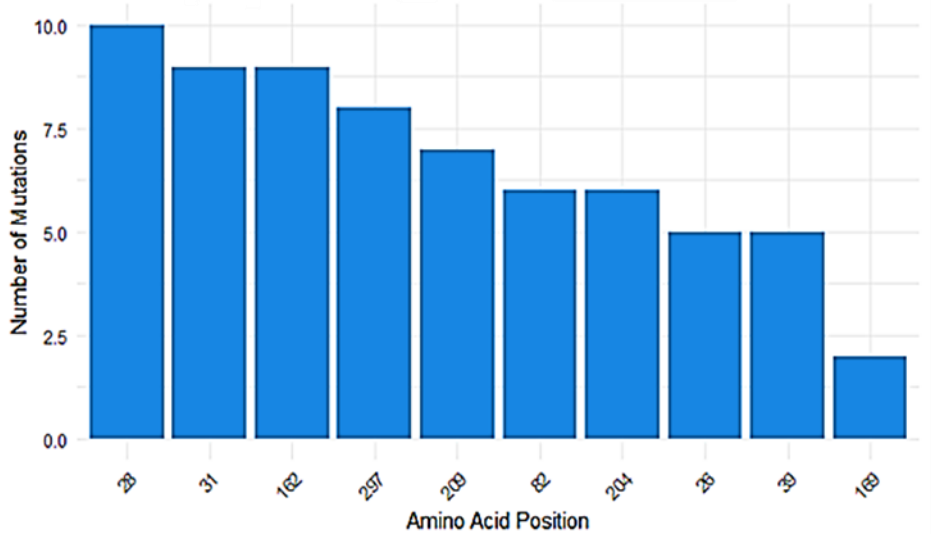

Fig. 6: Most frequently mutated positions in *B. longum* L-ASNase

4. DISCUSSION

L- asparaginase (L-ASNase) has emerged as a crucial enzyme in cancer therapeutics, particularly for acute lymphoblastic leukemia (ALL), due to its ability to deplete asparagine in serum and selectively target cancer cells dependent on external asparagine sources (Díaz-Barriga et al., 2021). Despite its clinical success, conventional L-ASNase treatments face limitations including immunogenicity, short half-life, and variable efficacy across different tumor types. These challenges highlight a significant research gap in enhancing the enzyme's therapeutic properties through structural modifications while maintaining its catalytic efficiency. The microbiome represents an underexplored source of potentially novel L-ASNase

variants with unique properties. While extensive research has been conducted on *E. coli*-derived L-ASNase, far less attention has been given to enzymes from commensal microorganisms like *L. lactis* and *B. longum*, which might offer improved pharmacological profiles due to their evolutionary adaptation to the human gut environment.

The present study addresses this gap by employing computational approaches to investigate how targeted mutations in these microbial L-ASNases might enhance their substrate binding and potentially their therapeutic efficacy. The analysis of *E. coli* L-ASNase revealed that four specific mutations (GLY198CYS, GLY198LEU, LEU190LYS, and PRO194ALA) modestly improved binding affinity from -5.8 kcal/mol in the wild-type to -5.9 kcal/mol in the mutants. These findings are particularly significant when considering the structural implications of each substitution. The GLY198 position appears to be a critical modulator of substrate binding, where the introduction of side chains through cysteine or leucine substitutions likely stabilizes the binding pocket architecture. The native glycine, lacking a side chain, provides flexibility that may not be optimal for substrate positioning. By introducing defined side chains at this position, we potentially created a more complementary binding surface for L-asparagine. The LEU190LYS mutation represents a substantial physicochemical change, introducing a positive charge into a previously hydrophobic region. This modification suggests that electrostatic interactions may play a more significant role in substrate recognition than previously acknowledged. The positively charged lysine could form favorable interactions with the carboxylate group of asparagine, enhancing binding stability. The PRO194ALA substitution is particularly intriguing from a structural dynamics' perspective. Proline residues are known to impose rigid constraints on protein backbone conformation. By replacing proline with the more conformationally flexible alanine, this mutation is likely altering the local dynamics of the active site, potentially facilitating improved substrate access or product release without compromising binding affinity. This finding aligns with previous research suggesting that optimizing enzyme dynamics can enhance catalytic efficiency without increasing substrate binding strength (Fig 7).

Fig. 7: Mutated active site for each 3 organisms tested in our study, highlighted with red, figure generated using CB-Dock2 server. *B. longum* (A), *E. coli* (B) and *Lactococcus* (C).

The analysis of *L. lactis* L-ASNase revealed a striking clustering of mutation effects in the 290-298 region, with position 295 (ASN295) demonstrating sensitivity to substitutions. This clustering suggests that this region constitutes a functionally important domain, likely involved in substrate recognition or catalytic activity. The observation that multiple positions within this region (particularly 295, 263, 172, and 298) tolerate various mutations while maintaining similar binding energies indicates that this enzyme may possess greater structural plasticity than its *E. coli* counterpart.

The consistent binding energies across diverse amino acid substitutions ranging from charge alterations (ASN295GLU, ASN295LYS) to steric modifications (PHE266LEU, ASN295MET) suggest that the L. lactis enzyme employs multiple, potentially redundant mechanisms for substrate recognition (Fig 8).

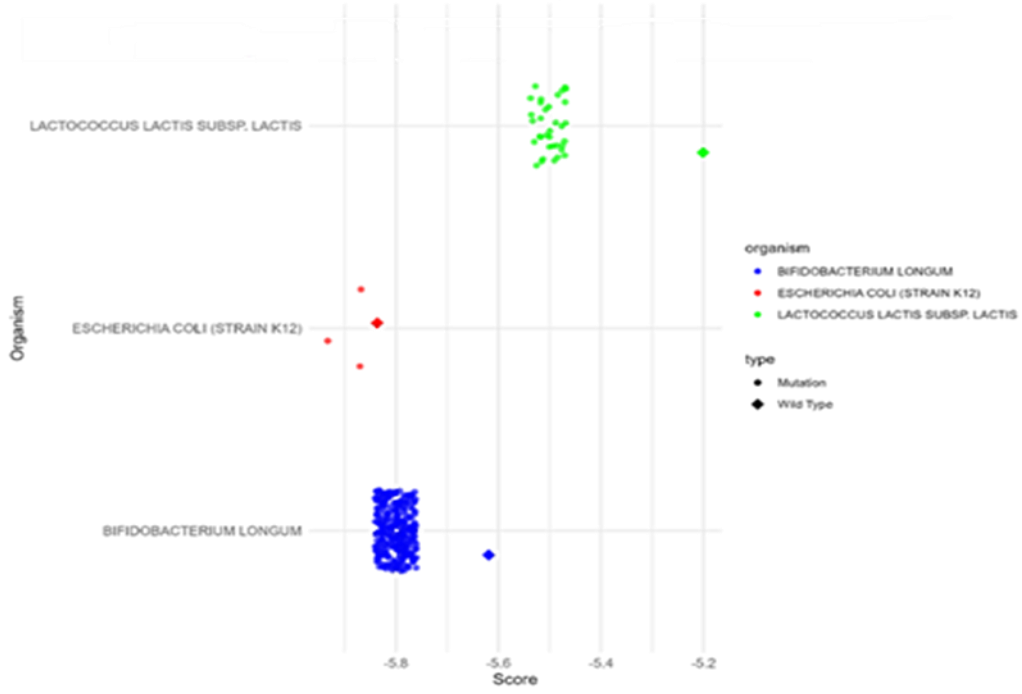

Fig. 8: Summary mutation score by each organism and the positional from each score with wild type

This functional redundancy might explain the enzyme's resilience to single-point mutations. The heatmap analysis further supports this conclusion by demonstrating uniform binding energies across diverse range of mutations, indicating that no single amino acid property dominates the binding interaction. The mutations affecting hydrogen bonding capabilities (SER263ALA, THR171CYS) provide insight into the role of specific non-covalent interactions in substrate recognition. While these substitutions did not dramatically alter binding energies, they likely influence the specificity and stability of enzyme-substrate complexes. Such modifications could be leveraged to fine-tune the enzyme's substrate preference or reaction kinetics for therapeutic applications.

Perhaps the most remarkable finding was observed in *B. longum* L-ASNase, where all tested mutations uniformly reduced binding affinity from -5.6 kcal/mol in the wild-type to -5.8 kcal/mol in the mutants. This consistent decline suggests an evolutionarily optimized active site where any deviation from the native sequence compromise's function. The uniformity of this effect across diverse amino acid substitutions, regardless of their physicochemical properties, indicates that the *B. longum* enzyme's active site architecture is exceptionally fine-tuned for its substrate. The affected positions (26, 28, 31, and 39) likely represent critical enzyme specificity and efficiency. Their low tolerance to mutation suggests they may be involved in precise substrate positioning or maintaining the active site's structural integrity. This evolutionary conservation aligns with *B. longum's* specialized niche in the gut microbiome, where efficient nitrogen metabolism through asparagine hydrolysis would confer a significant selective advantage. The apparent rigidity of the B. longum enzyme contrasts with the relative plasticity observed in *L. lactis* L-ASNase, highlighting how different evolutionary pressures can shape enzyme structure-function relationships even for homologous proteins. This finding has important implications for enzyme engineering strategies, suggesting that *B. longum* L-ASNase might require more sophisticated approaches, such as directed evolution or computational design of multiple coordinated mutations, to enhance its therapeutic properties.

This study addresses several key aspects of the identified research gap. First, by systematically comparing L-ASNases from three distinct microbial sources, we have expanded our understanding beyond the well-studied *E. coli* enzyme to include commensal organisms with potential therapeutic advantages. Identifying specific mutations that modestly enhance binding in *E. coli* L-ASNase provides concrete targets for experimental validation and potential enzyme engineering. Second, our comprehensive analysis of the structure-function relationship in these enzymes has revealed source-specific differences in mutational tolerance and binding mechanisms. The exceptional sensitivity of *B. longum* L-ASNase to mutations suggests this enzyme may be naturally optimized for its function, potentially offering superior stability or kinetic properties in therapeutic applications without extensive modification.

Third, the identification of key regions and specific residues that influence substrate binding across all three enzymes provides valuable insights for rational enzyme design. Future studies can target multiple residues simultaneously to achieve synergistic improvements in enzyme performance by understanding how different amino acid substitutions affect binding

affinity. Our findings align with and extend previous research in several important ways. The modest improvement in binding affinity observed for *E. coli* mutations is consistent with prior studies suggesting that L-ASNase has evolved near-optimal catalytic efficiency, with limited room for enhancement through single amino acid substitutions. However, our identification of specific positions that tolerate or even benefit from mutation offers new opportunities for fine-tuning enzyme properties.

The clustering of mutation effects in *L. lactis* L-ASNase around positions 290-298 corresponds with previous structural analyses identifying this region as part of the substrate binding pocket. Our more detailed characterization of positional sensitivities within this region provides novel insights into the architectural determinants of substrate recognition and binding. The remarkable conservation observed in B. longum L-ASNase aligns with emerging recognition of commensal microbiome species as potentially valuable sources of therapeutic enzymes. Previous studies have suggested that gut-adapted microorganisms may produce enzymes with superior stability under physiological conditions. The study finds that *B. longum* L-ASNase appears highly optimized in its wild-type form supports this hypothesis and suggests that this enzyme might offer advantages over *E. coli*-derived variants currently used in clinical settings.

In contrast to previous studies focusing primarily on enhancing catalytic efficiency, this study emphasizes the multifaceted aspects of enzyme optimization, including substrate binding affinity, structural plasticity, and evolutionary conservation. This comprehensive approach provides a more nuanced understanding of how different microbial L-ASNases might be tailored for specific therapeutic applications. While the binding energy changes observed in our study were modest, they provide crucial insights into the structure-function relationships governing L-ASNase activity. These findings lay the groundwork for more sophisticated enzyme engineering strategies that could ultimately lead to improved cancer therapeutics with enhanced efficacy and reduced side effects.

5. CONCLUSION

The study has drawn insightful conclusions on the structural and functional implications of amino acid replacements in L-ASNase, more particularly from Lactococcus lactis and Bifidobacterium longum. Through critical analysis of differences in binding affinity, active site clustering, and frequency of mutation, signature positions have been identified with determinative roles in enzyme activity, stability, and catalytic efficiency.

Furthermore, the mutation analysis shows that although all the substitutions tested slightly increase binding energy, their overall impact on enzyme stability, catalytic turnover, and substrate specificity need to be validated experimentally. In addition, this study demands particular enzyme engineering strategies for optimizing L-ASNase for industrial and therapeutic applications. Follow-up research would entail investigating structural flexibility, determining the best combination of mutations for the optimum ratio, and validating silico optimizations with biochemical assays. With a better understanding of such molecular optimizations, more efficient enzyme variants for medical and biotechnological applications can be engineered.

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