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Molecular analysis of Kefiran exopolysaccharide: Interactions with vitamins, amino acids, monosaccharides, lipids, and Toll-like receptor 4

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Abstract

Kefir grains are composed of a consortium of microorganisms, which are enveloped in a matrix of exopolysaccharides called kefiran, with an almost equal proportion of D-glucose and D-galactose, produced by lactic acid bacteria of the genus Lactobacillus. Associated with a diversity of proteins and lipids, kefiran supports the symbiotic cellular structure and protects it against external influences, such as nutrient deficiencies, dehydration, bacteriophages, toxicity, and osmosis. Kefiran has attracted the attention of the scientific community due to its functional properties, and its role in modulating the immune system is the most studied and has potential for exploration. However, the biopolymer has a complex molecular structure and its physical and chemical properties still require investigation. The present study addresses the interaction between the kefiran tetramer and various compounds: trivalerate triacylglycerol; monosaccharide — D-glucose; fat-soluble vitamins such as vitamin A — retinol and vitamin D — cholecalciferol; water-soluble vitamins such as vitamin B2 — riboflavin and vitamin C — ascorbic acid; and amino acids such as positive side chain histidine, negative aspartate, polar asparagine, and hydrophobic isoleucine. From its molecular properties and experimental results from the literature, we discuss the role of kefiran in acting similarly to the carbohydrate portion of lipopolysaccharides in the Toll-like receptor and myeloid differentiation factor 2 system, activating the immune system.

Keywords: kefir; kefiran; exopolysaccharide; nutrients; molecular dynamics; TL receptors.

Practical Application: A set of molecular properties is presented and discussed for kefiran exopolysaccharide. This bioactive has a complex structure, being modeled here as the monomer and tetramer of D-glucose and D-galactose glycosidic bonds. Thus, the kefiran–nutrient interactions can assist in explaining the experimental — physical, chemical, and biological — properties of this biopolymer, as explored here as an activator of the immune system via Toll-like receptor 4.

1 INTRODUCTION

1.1 Kefir system: Fermented milk, grains, and health benefits

The kefir drink originates from the Caucasian Mountains, the extreme southeast of Europe between the Caspian Sea and the Black Sea, with the word "kefir" originating from the Turkish term "keif," which means "well-being". It is estimated that the cultivation of this drink began around 2000 BC, being widely spread among generations of people and tribes in that region (Rosa et al., 2017). Currently, it is known that this drink has been produced and consumed on all continents, given its functional potential as a fermented food product, traditionally made through the fermentation of milk with kefir grains (González-Orozco et al., 2022), being the subject of study in recent years.

The fermented product is composed of bacteria and yeasts that proliferate in a microbial association forming a macrostructure called "kefir grains," such grains are made up of lactose fermenting and non-fermenting yeasts (Ibacache-Quiroga et al., 2022). In short, for the formation of grains, the self-aggregation of *Lactobacillus kefiranofaciens* and *Saccharomyces* spp. initially occurs, forming small granules. Then, the *Lentilactobacillus kefiri* attach themselves to the surface of the granules, aggregating with other organisms and components present in the milk, generating the visually characteristic macrostructure. Due to the fermentation process of releasing lactic acid and acetic acid, resulting from the bioconversion of lactose, the drink has an acidic flavor (González-Orozco et al., 2022); the cultivation of microorganisms can be done using water and sugar, or milk, and is considered a probiotic food (Abraham & De Antoni, 1999). Given the beneficial properties for health reported in

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the literature, and through empiricism, the food industry has proposed and marketed a series of products based on this fermented drink, which include yogurts, creamy cheeses, açaí pulp, protein ice creams, and associations with jellies, among others (Sperotto et al., 2017; Zottmann da Silva & Weschenfelder, 2020). Its consumption has become popular all over the world, and for this reason, it has been called the "yogurt of the 21st century" (Gentry et al., 2023).

The other important component in this kefir system, the grains, have unique characteristics, such as softness, gelatinous appearance, and irregular shape, ranging in size from 0.3 to 3.0 cm in diameter. The complex microbial consortium present in kefir grains can produce a variety of metabolites and differs from other fermented products as they comprise a specific and complex mixture of bacteria that produce lactic acid, acetic acid, and various bioactive compounds, in addition to lactose-fermenting and non-lactose-fermenting yeasts, which coexist in symbiotic association (Ganatsios et al., 2021; Rosa et al., 2017). As well as the liquid component of the drink, grains are also known to have health-promoting effects, including benefits in reducing cholesterol levels, preventing cardiovascular disease, and antioxidant action (Tan et al., 2020).

Through the milk fermentation process, several compounds are produced that give kefir its characteristic aroma and flavor. Many of these compounds have nutraceutical properties that, together with a balanced diet, generate benefits such as increased growth of bifidobacteria and lactobacilli in the intestinal microbiota, stimulation of the immune system, antimicrobial activity against pathogens, and possible antitumor actions (Ahmed et al., 2013). Regular intake of this product is recognized for promoting intestinal health, controlling serum glucose and cholesterol levels, as well as offering other benefits given its probiotic nature (da Silva & Okura, 2021; Gomes et al., 2020). These benefits are associated with microorganisms preserving the probiotic function, as well as the activities of exopolysaccharides (EPSs) and the peptides produced during the fermentation process. Furthermore, regular and constant intake of kefir has been linked to improved digestion and lactose tolerance, antibacterial effects, hypocholesterolemic effects, control of plasma glucose, antihypertensive effects, anti-inflammatory effects, and antioxidant activity, as well as anticancer, antiallergic, and healing properties, among others (Rosa et al., 2017).

1.2 The exopolysaccharide kefiran: Structure and health benefits

Exopolysaccharides are biopolymers secreted by bacteria and fungi, natural components of their habitat, and can be obtained from plant, animal, or microbial sources. Microbial EPSs perform vital ecological and physiological functions, acting to protect microorganisms in adverse environments, in addition to promoting adhesion to solid surfaces, and facilitating the formation of biofilms (Tan et al., 2020). These biofilms are complex structures that provide a series of advantages to microorganisms, such as protection against antimicrobial substances and antibodies, in addition to serving as a selective barrier to the diffusion of nutrients into and out of the cell, contributing to cellular function. These properties make EPS targets of interest in several areas, including biotechnology, medicine, and the food industry (Barcelos et al., 2020; Shankar et al., 2021; Wang et al., 2019).

In 1967, Riviére et al. first isolated an unknown EPS called "kefiran" from kefir grains, which was classified as a food biopolymer. Initial characterization of kefiran revealed that this natural polymer has a molecular mass range of $1.0x10^4$–$6.6x10^6$ Da, depending on the carbon source used. This molecular weight range is comparable to other natural polysaccharides, such as starch and chitosan, for example. In general, kefiran is a water-soluble microbial EPS composed of glucose and galactose, formed by monomers linked by O-glycosidic bonds, and can be classified as heteropolysaccharides due to the set and diversity of monosaccharides in its structure (Barcelos et al., 2020; Wang et al., 2019), being suggested by Kooiman et al. in 1968, after methylation studies and oxidation analysis, and being corroborated by Mukai et al. in 1987 and 1990 (Mukai et al., 1990; 孝夫向井 et al., 1988). In the late 1990s, Micheli et al. (1999) proposed a defined structure for each monomeric unit composed of six hexoses, with the ratio maintained at 1:1 between glucose and galactose, in beta-glycosidic bonds for the most part, as shown in Figure 1.

The main structure of the polysaccharide is composed of D-glucose and D-galactose, with a D-glucose branch. The majority composition of beta-glycosidic bonds prevents hydrolysis by digestive enzymes in the human gastrointestinal tract; however, kefiran can be fermented by bacteria (Gentry et al., 2023). It is suggested that, through the association between a variety of proteins and lipids, kefiran supports the symbiotic cellular structure, protecting it from external influences, preventing nutrient deficiencies, dehydration effects, bacteriophage actions, chemical toxicity, and osmosis. In this sense, kefiran has been directly associated with the bioavailability of nutrients, enhancing the nutritional aspects of foods.

Furthermore, the literature has shown that kefiran has antimicrobial, healing, antibacterial, antitumor, antioxidant, anti-inflammatory, and biocidal properties, and some capacity to modulate the intestinal immune system, in addition to effects on reducing blood pressure. The functional properties associated with this biopolymer, such as stimulation of the immune system and antimutagenic activity, have aroused interest in the scientific community. Studies have shown the effects of kefiran on lipids, blood pressure, and blood glucose levels, where significant inhibition of blood pressure (Gentry et al., 2023), reduction of blood cholesterol levels, and reduction of blood glucose levels were observed. Another important and described role of kefiran is in immunomodulation. Kefiran is capable of inducing the expression of IL-10, an inhibitory cytokine in inflammatory processes, and increasing the phagocytic activity of dendritic cells. In addition, it appears to prevent the increase in the levels of inflammatory mediators induced by lipopolysaccharide (LPS) in monocytes. This immunomodulatory role was also observed in the lamina propria of the large intestine of animals treated with kefiran, where the levels of IgA+, IgG+, IL-4+, IL-10+, IL-6+, and TNFα+ increased during the 7 days of treatment (Vinderola et al., 2006).

Due to its versatility, the application of kefiran for the delivery of biopharmaceutical drugs and other biomedical applications is also being discussed. These results suggest that the kefiran biopolymer has the potential to act as a functional food

or in formulations with therapeutic applicability. Considering the extensive discussion in the literature about the versatility of kefiran in biological and biotechnological applications, in addition to its pharmacological effects, this work presents a molecular description of the interaction of this EPS with nutrients — vitamins, amino acids, sugar, and lipids using molecular dynamics simulations. A discussion of its immunological effects from a molecular point of view is also presented.

2 MATERIALS AND METHODS

2.1 Molecular dynamic simulations: Kefiran–nutrient interactions

This is a powerful technique for evaluating molecular interactions (intra and inter), computing short/long-range interactions, and understanding how atoms interact over time, especially used for proteins, macromolecules, and solution systems (Borges et al., 2023; Lescano et al., 2021; Oliveira & Caires, 2019). Different force fields and programs can be used to perform the molecular dynamics (MD) simulations as they have potential energy functions similar to those presented in Equation 1. In this equation, V_{total} is the sum of intramolecular (bonded) and intermolecular (non-bonded) potentials, such as covalent bonds, angles formed by three adjacent atoms covalently bound, dihedral torsion to a set of four atoms, Lennard–Jones potentials for pairs of atoms interacting by van der Waals forces, and electrostatic potential, which describes the interactions between charged particles:

$$V_{total} = \frac{1}{2}\sum_{bonds} k_b(r-r_0)^2 + \frac{1}{2}\sum_{angles} k_\theta(\theta-\theta_0)^2 + \frac{1}{2}\sum_{dihedrals} k_\omega[1+cos(n\omega-\gamma)] + \sum_{pairs} 4\varepsilon_{ij}\left[\left(\frac{\sigma_{ij}}{r_{ij}}\right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}}\right)^{6}\right] + \sum_{pairs}\frac{q_i q_j}{4\pi\varepsilon_0 r_{ij}} \quad (1)$$

To calculate the interactions between kefiran and nutrients, two systems were built according to structures presented in Figure 2: System i consists of the kefiran tetramer in water, whereas system ii includes the kefiran tetramer with nutrients — vitamins, amino acids, saccharides, and triacylglycerol, containing twenty molecules of each. The initial configurations of simulation boxes were built using Packmol (Martínez et al., 2009). All systems containing the kefiran tetramer, with and without nutrients, were equilibrated by performing 10,000 steps of Conjugate Gradient (CG) minimization followed by 60 ns of MD simulations. Production runs were performed for each system in the NPT (number of particles, pressure, and temperature constants) ensemble at 1 atm and 298.15 K. The pressure was controlled using a Langevin barostat with a period of 200 fs, 100 fs decay, and a piston temperature of 293.15 K. A constant temperature was maintained using a Langevin bath with a 10 ps 1 damping coefficient. The Chemistry at HARvard Macromolecular Mechanics (CHARMM) force field was used for all molecules (Nauss, 2000). Simulations were performed using the NAMD program (Nelson, 1995), and figures/analyses were produced using VMD, Pymol, and MDAnalysis programs (Humphrey et al., 1996).

2.2 Molecular docking: Immune system activation

Studies have associated kefiran with improving anti-inflammatory response and immune system activation. The interactions with Toll-like receptors (TLR) are a reliable behavior reported to the kefiran mechanism for immunological benefits as previously suggested (Bahari et al., 2020). The recognition of diverse LPS molecules by the TLR-4 and myeloid differentiation factor 2, MD-2, heterodimer (Park et al., 2009a) suggests possible interactions with the polysaccharide kefiran. In this sense, molecular docking calculus was performed to evaluate

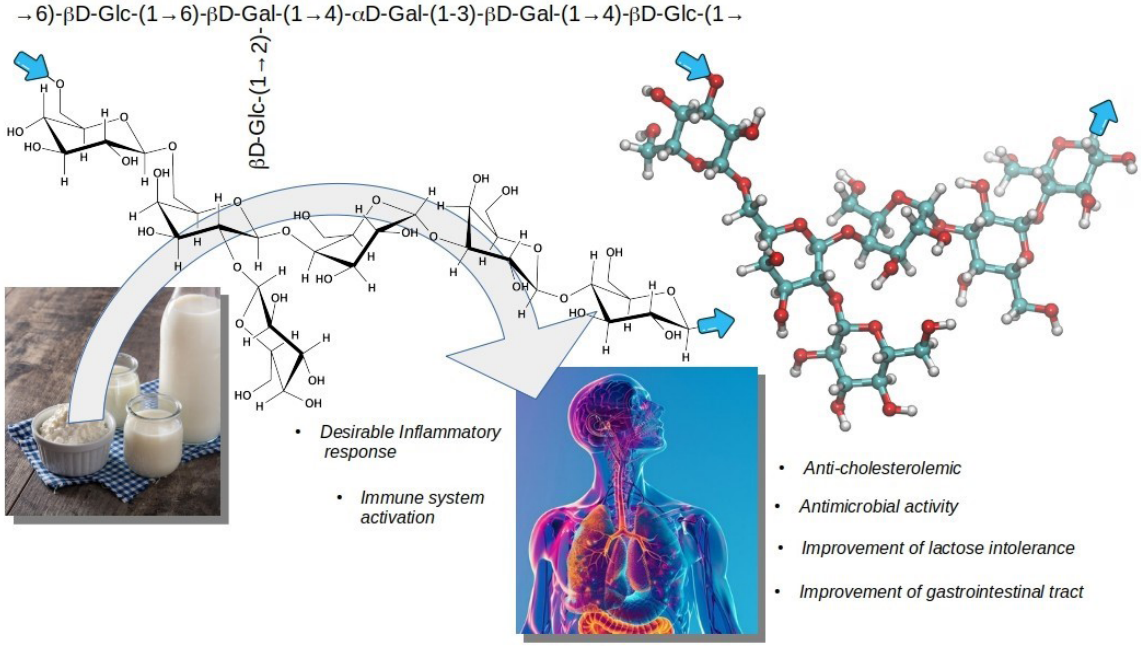

Figure 1. Monomeric structure of the kefiran exopolysaccharide containing six monosaccharides and their alpha and beta-glycosidic linkages between D-glucose and D-galactose (Micheli et al., 1999).

the complex TLR-4-MD-2-Kefiran system from the molecular point of view, as shown in Figure 3.

The binding modes of kefiran with the TLR4-4-MD-2 complex were investigated using a molecular docking method. This compound was docked into the catalytic site of the receptor using the DockThor program (de Magalhães et al., 2014). The molecular docking was established in a cubic grid box (Δx, Δy, and Δz of 30, 30, and 30 Å), centered at coordinates x, y, z 26.0200, -10.3150, 13.5680 and discretization of the energy at 0.31 Å. The crystal structure of TLR4 complexed with MD-2 was obtained from the Protein Data Bank [PDB ID: 3FXI (Park et al., 2009b)]. We choose one of the subunits of the homodimer to investigate the ligand–receptor interactions at the binding site domain. The parameters are referred to as defaults in DockThor, and the structures with positional root mean square deviation (RMSD) of up to 2 Å were clustered together.

3 RESULTS AND DISCUSSION

The biological behavior of the biopolymer kefiran has been explored from several approaches, including interactions with nutrients and protein receptors that justify its experimental and empiric health benefits. To understand these two possible molecular explanations, a time dependence of kefiran interactions with vitamins, amino acids, saccharides, and lipids is discussed, followed by an exploration of its possible anchorage to the TLR4-MD-2 receptors.

3.1 The preferential kefiran–nutrient interactions

The kefiran mobility was analyzed by RMSD, as shown in Figure 4A. In an aqueous solution, kefiran was stabilized with the lowest RMSD values compared to an aqueous nutrient solution — a comparison between gray lines. The presence of nutrients promotes changes in the tetramer biopolymer reaching RMSD values higher than 10 Å at the end of 60 ns of simulation. It is explained by the short kefiran–nutrient interaction emerging and the possible loss of own kefiran interactions. The nutrients presented different behaviors with high displacement for asparagine and medium displacement for histidine, aspartate, isoleucine, ascorbic acidic, and glucose. In general, this find suggests a high translation of these nutrients around the kefiran tetramer without a specific place of anchoring. The most relevant vitamins seem to be more stabilized in a centered coordinate of atoms, showing less displacement in the simulation box. Similarly, the lipid model, trivalerate glycerol, was well stabilized, suggesting a possible anchoring on the biopolymer surface. In fact, Figure 4B shows the disposition of some molecules of lipids very close to kefiran, a phenomenon observed during all the simulation trajectories.

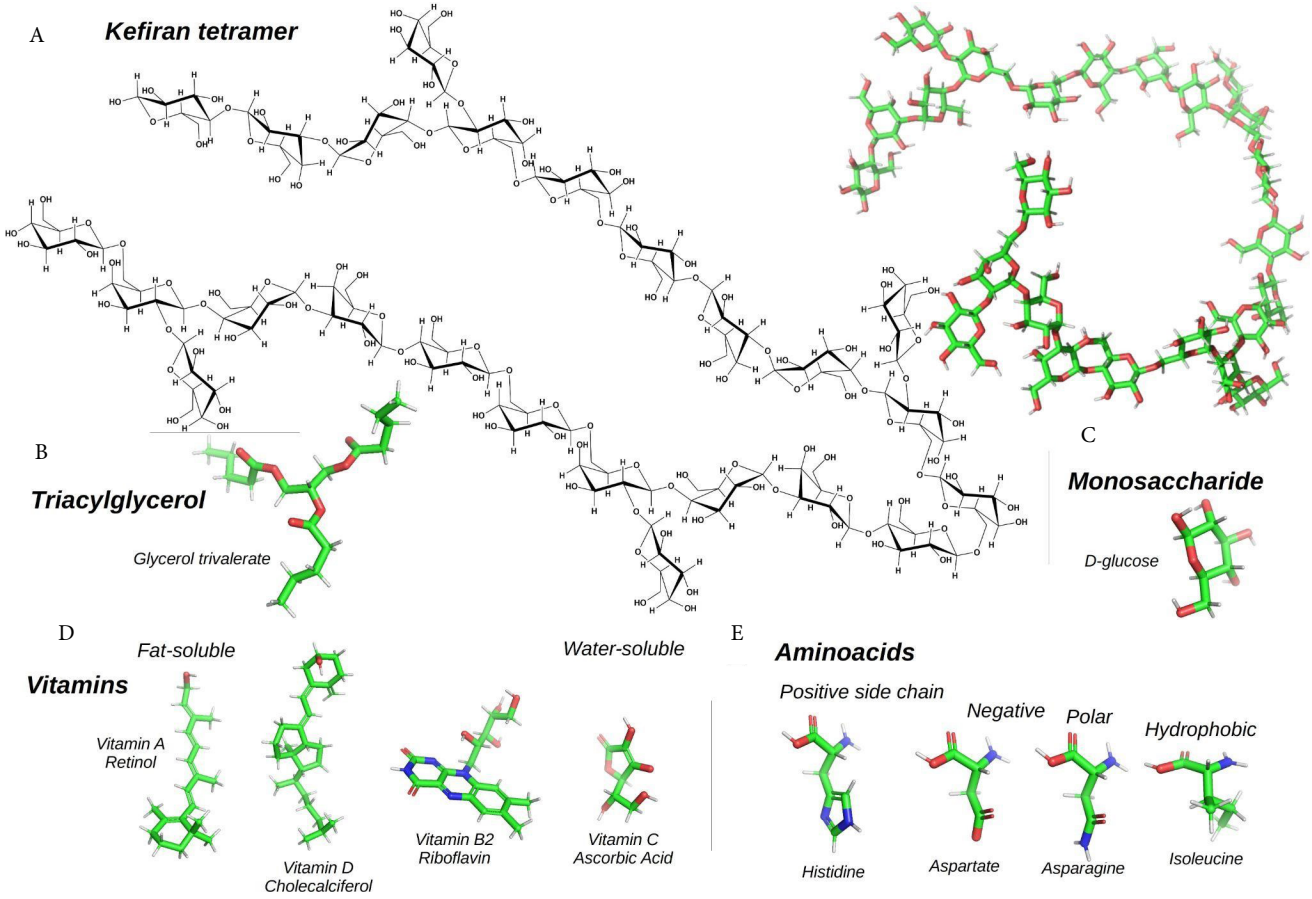

Figure 2. Structures modeled for simulations: (A) Kefiran tetramer; (B) triacylglycerol — glycerol trivalerate; (C) monosaccharide — D-glucose; (D) fat-soluble vitamins: vitamin A — retinol and vitamin D — cholecalciferol; water-soluble vitamins: vitamin B2 — riboflavin and vitamin C — ascorbic acid; and (E) amino acids: positive side chain histidine, negative aspartate, polar asparagine, and hydrophobic isoleucine.

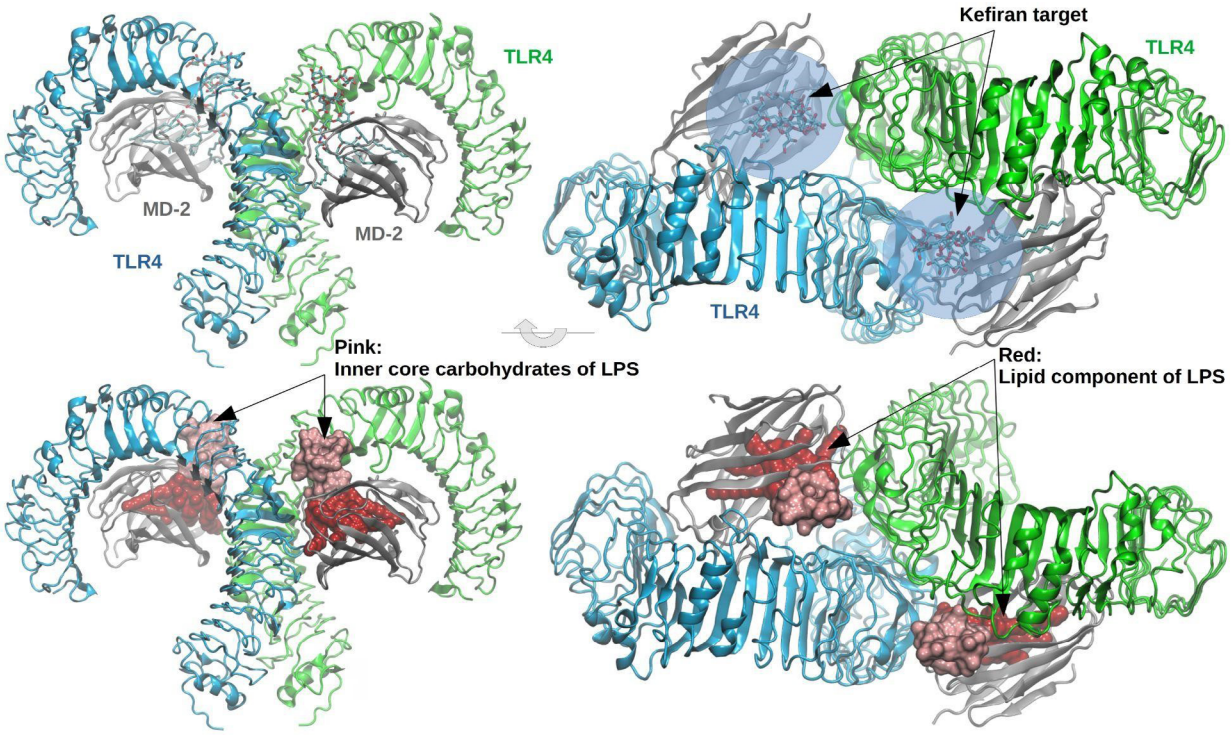

Figure 3. Overview of Toll-like receptor (TLR) 4 and myeloid differentiation factor 2 (MD-2), highlighting the lipopolysaccharide, LPS, binding site (Park et al., 2009a).

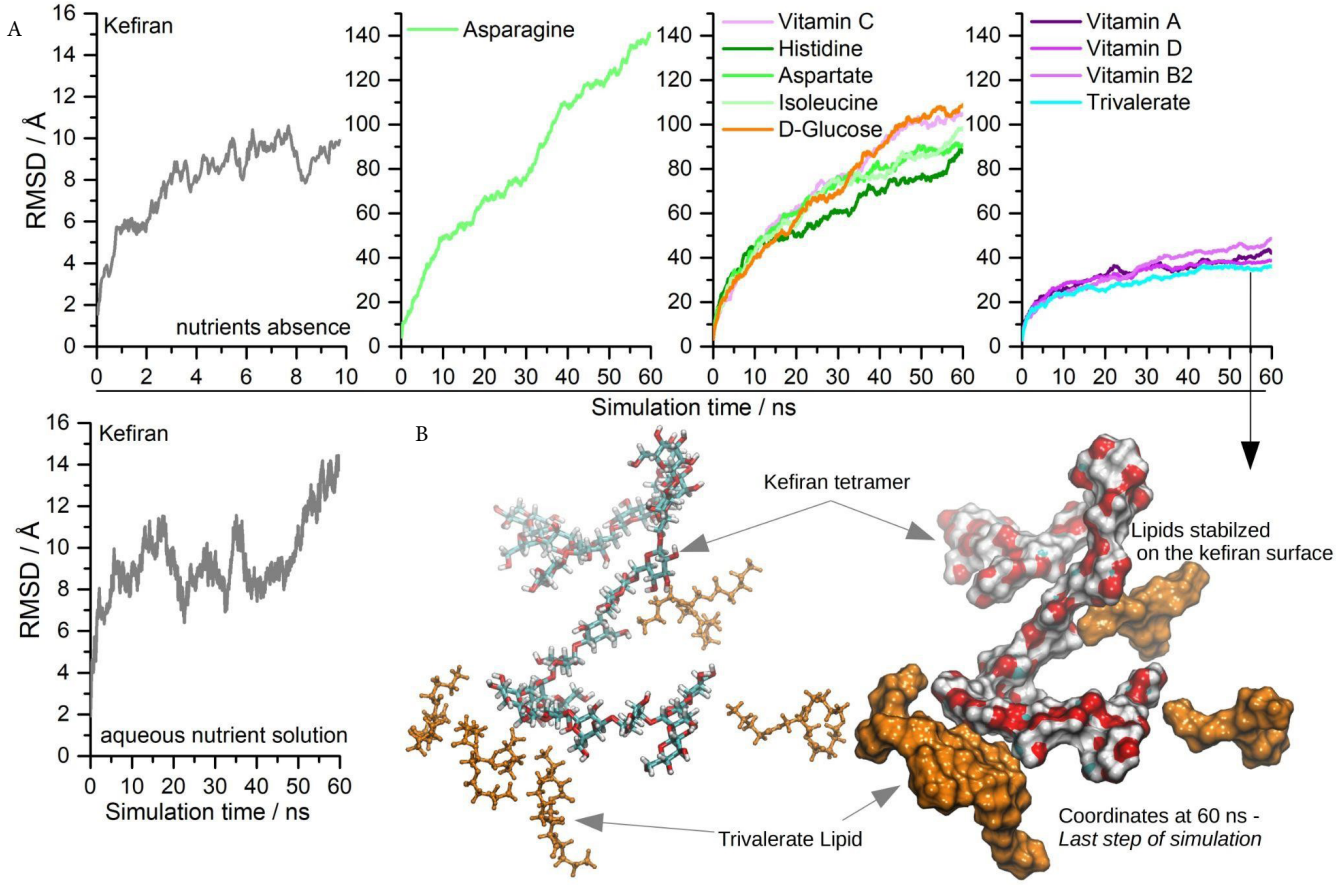

Figure 4. (A) RMSD of kefiran tetramer and nutrient components in the simulation trajectory. (B) Qualitative accumulation of the lipid glycerol trivalerate around the tetramer, the most stabilized nutrient in the simulation.

Distribution functions are adequate to understand the accumulation or repulsion of a solvent around a solute, especially minimum distance distribution functions (MDDFs), ideal for non-spheric solutes (de Oliveira & Martínez, 2019; Martínez & Shimizu, 2017). An overview of the preferential interactions between the kefiran tetramer and nutrients shows a smooth displacement of water around the polysaccharide to accommodate especially hydrophobic compounds, as shown in Figure 5A. An exception of vitamin A, retinol, the other nonpolar nutrients are energetically allocated close to the polymer surface, as observed for vitamin D, cholecalciferol, and amino acid isoleucine. Surprisingly, the lipid model, glycerol trivalerate, is shown in Figure 5B with the highest accumulation around the kefiran polymer. The kefiran–lipid interaction is interesting; from this finding, it is possible to present the basis for understanding the related effect on immune activity response due to its similar LPS structure, with specific interaction in the TLR4-MD-2 receptors, for instance. In addition, solvation analyses of solvent–kefiran accumulation at 3Å from the polymer surface, Figure 5C, reinforce the MDDF findings, i.e., vitamins are frequently found close to the kefiran tetramer, and the lipid trivalerate is frequently and quantitatively close to the kefiran, as expected for a similar LPS structure.

3.2 Kefiran and TLR4-MD-2 interaction

Kefiran has been studied due to its functional properties, such as non-toxicity, antimicrobial activity, important nutritional value, and other promising characteristics (Tan et al., 2020). However, of the various biological effects of kefir and kefiran highlighted in the literature, their role in modulating the immune system is the most studied and elucidated (Bahari et al., 2020; Gentry et al., 2023). Kefiran is known to modulate the cytokine profile (IL-4, IL-10, IL-6, IFNγ, and TNFα) and immunoglobulin IgA in BALB/c mice after 7 consecutive days of oral administration (Vinderola et al., 2006). *In vitro* assays show that kefiran (50 μg/mL) is capable of reducing responses mediated by Toll-like receptor 4 (TRL4) activation by the agonist LPS in monocytes derived from peripheral blood mononuclear cells. In this sense, kefiran reduces the activity of LPS in inducing the production of cytokines IL-1β and TNF-α in monocytes. On the other hand, it appears to induce the expression of IL-10, an inhibitory cytokine in inflammatory processes, in dendritic cells derived from monocytes. Such actions reflect the improvement of the innate immune function of these cells, with an increase in their phagocytosis capacity, a crucial aspect of

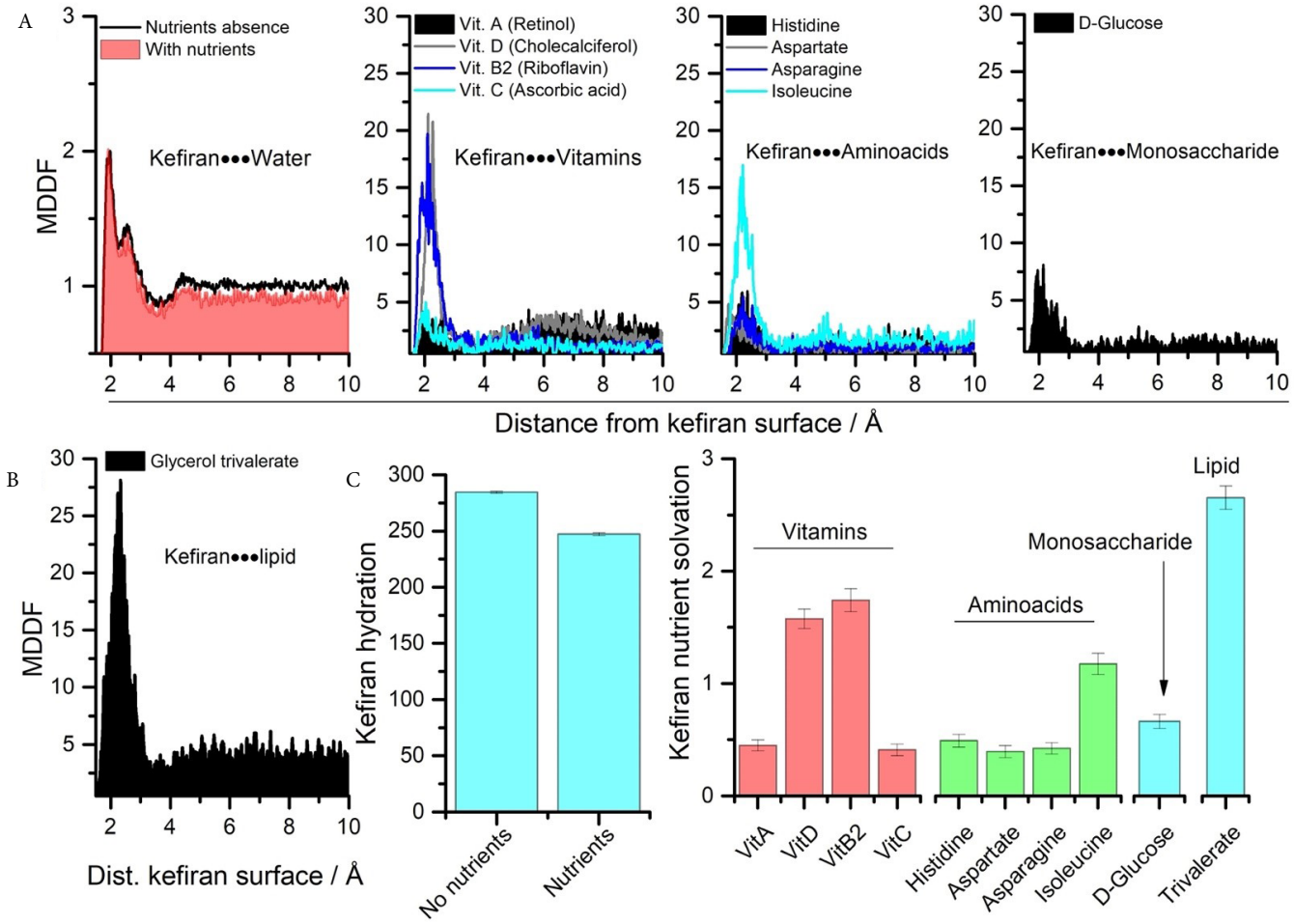

Figure 5. Preferential distribution of nutrients around the kefiran tetramer surface according to the minimum distance distribution function (de Oliveira & Martínez, 2019; Martínez & Shimizu, 2017): (A) kefiran and water, vitamins, amino acids, and monosaccharide; (B) MDDF kefira–lipid; and (C) kefiran solvation at 3 Å from the polymer surface by water, and simulated nutrients.

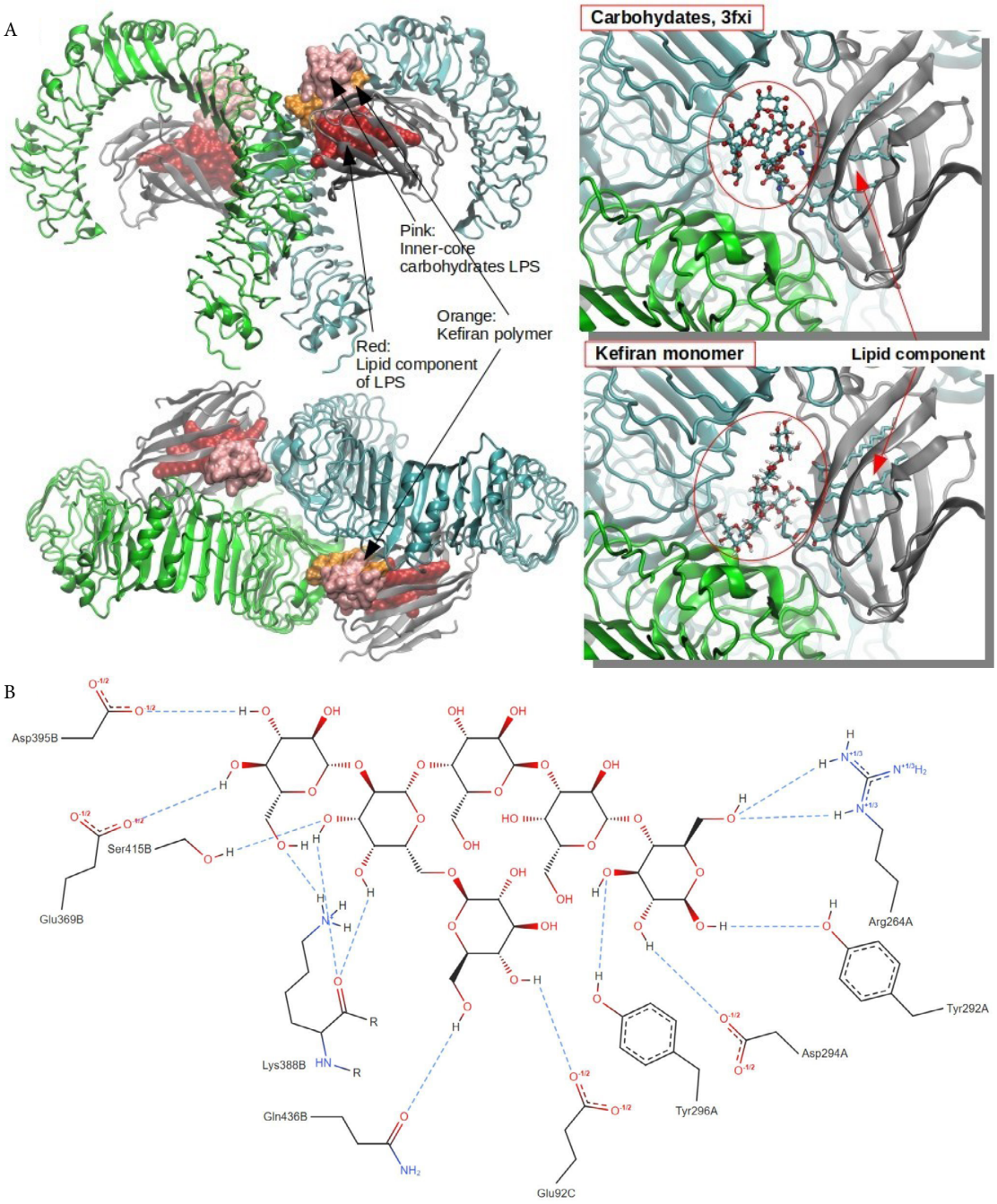

Figure 6. TLR-4-MD-2 complexed with kefiran polysaccharides. (A) Similar position observed for both the carbohydrate component of LPS and kefiran. (B) Sampled residues that stabilize kefiran in the carbohydrate pocket such as Tyr296 and Asp294.

the defense against pathogens (Bahari et al., 2020; Park et al., 2009a). Figure 6 shows how kefiran can interact with the TLR4-MD2 system, highlighting the residues that stabilize the ligand in the accommodation region of the carbohydrate part of LPS. The similarity between the binding modes allows us to infer its regulatory role similar to that expected for LPSs.

Kefiran appears to exert immunostimulatory effects both *in vitro* and *in vivo* because polysaccharides such as kefir can stimulate the production of specific antibodies without the recruitment of T cells or induction of immunological memory (Kalka-Moll et al., 2002). In simple terms, kefiran acts as a weak provocateur, but not to the point of inducing immunological memory and immunoglobulin class switching; it mildly stimulates the immune system. Some studies report that this ability may be associated with its binding to TLRs, especially TLR2 and TLR4 (Bahari et al., 2020).

4 CONCLUSIONS

From molecular modeling analysis, it was possible to establish an interaction profile of kefiran with common nutrient

molecules such as vitamins, amino acids, monosaccharides, and lipids. The analyses allow us to infer a favorable interaction between kefiran and the lipid–glycerol trivalerate, in addition to strong interactions with the fat-soluble vitamin cholecalciferol, in addition to the amino acid isoleucine. This property of interacting with nutrients with a hydrophobic profile makes it possible to interpret that kefiran can bind to the interaction region of the carbohydrate portion of LPS in the TLR4-MD2 system, similarly to that expected for LPS, thus helping to explain its observed functional properties.

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