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Synthetic Mimotopes of Salivary Peptides against SARS-CoV-2 In Vitro

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Abstract

To replicate, SARS-CoV-2 requires its receptor-binding domain (RBD) motif in the Spike protein to interact with specific cellular receptors, such as angiotensin-converting enzyme 2 (ACE2), in human cells. Hence, we aimed to select high-affinity naturally expressed salivary peptides by bioinformatics to assess their potential to block the Spike-RBD/ACE2 interaction in vitro and to evaluate their SARS-CoV-2 antiviral performance. We designed a pipeline with 2,193 salivary peptides and performed molecular docking with 298 peptides using BLASTp. In silico molecular docking was evaluated using HPEPDOCK and validated by molecular dynamics with GROMACS-2020.3 against crystallographed Wuhan wild-type SARS-CoV-2 (SARS-CoV-2_{WT}) Spike-RBD, and the 4 best-performing peptides were redocked against Gamma, Delta, and Omicron Spike-RBD. These 4 selected peptides were synthesized, and antiviral activity was evaluated using VSV-eGFP-SARS-CoV-2 pseudotyped virus and SARS-CoV-2_{WT}. Finally, we evaluated the inhibition of cell death in SARS-CoV-2_{WT} and viral replication inhibition of Gamma and Omicron variants by synthetic mimotopes of salivary peptides by quantifying viral titers using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Salivary peptides SalivaPep1, SalivaPep2, SalivaPep3, and SalivaPep4 had higher binding energy to SARS-CoV-2_{WT}-Spike-RBD, and their effects were maintained against variants, suggesting salivary peptide interactions with SARS-CoV-2. The VSV-eGFP-SARS-CoV-2 infection inhibition rates of selected salivary peptides ranged from 67.5% to 37.6%, respectively, at about 100% cell viability. SalivaPep1, SalivaPep2, and SalivaPep3 reduced cell death resulting from viral replication against SARS-CoV-2_{WT}. Lastly, RT-qPCR showed up to a 74.9% decrease in viral copies against

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the Gamma variant and 62.5% against the Omicron variant by the treatment with SalivaPep3 and SalivaPep2, derived from salivary Ataxin-1 and Statherin, respectively. The presented bioinformatics workflow was profitable to select on-demand naturally occurring salivary peptides with confirmed antiviral properties in SARS-CoV-2_{WT}, Gamma, and Omicron variants by synthetic mimotopes of salivary peptides, with potential application to prophylactic, diagnostic and therapeutic strategies toward COVID-19.

Keywords: COVID-19, SARS-CoV-2 variants, Spike glycoprotein, molecular docking, antiviral agents, saliva

Introduction

SARS-CoV-2, the causative virus of COVID-19, has led to more than 37.5 million cases in Brazil and more than 775 million globally, with almost 7 million deaths (World Health Organization 2024). The disease varies from asymptomatic to severe respiratory failure and death (Nalbandian et al. 2021). COVID-19 detection is typically via reverse transcription polymerase chain reaction (RT-PCR) tests on nasopharyngeal swabs; however, a recent meta-analysis indicates that saliva presents similarity in SARS-CoV-2 RNA detection, providing a noninvasive detection of COVID-19 (Caixeta et al. 2023). Preventing infection by inhibiting viral replication remains the most effective strategy against new outbreaks (Monteil et al. 2020). Currently, only a limited number of antiviral drugs are licensed for COVID-19; however, their high cost and restricted availability hinder widespread application, particularly in low- and middle-income countries (Pan et al. 2022).

SARS-CoV-2, a member of the *Coronaviridae* family, is composed of the following structural proteins: spike protein (S), matrix protein (M), and envelope protein (E) (Lu et al. 2020). For virus entry into the host cells, the S receptor-binding domain (RBD) interacts with the host cellular receptor (Zawilska et al. 2021). The most well-characterized SARS-CoV-2 human receptor is the angiotensin-converting enzyme 2 (ACE2) (Oliveira et al. 2020). ACE2 receptors are highly expressed on oral mucosae, indicating susceptibility for SARS-CoV-2 infection (Huang et al. 2021).

Saliva plays a crucial role in innate immunity, providing a first-line defense through its antimicrobial and antiviral properties. Saliva contains a diverse array of antiviral bioactive molecules, including specific antibodies, enzymes, and naturally occurring peptides, which interact with viral surfaces to inhibit replication and reduce infectivity (Malamud et al. 2011). Although saliva presents recognized antiviral properties, several viruses are frequently detected in this oral biofluid. This paradox may arise from the limited potency and specificity of antiviral salivary components as well as individual variations in the concentration and activity of these proteins in saliva, which can influence susceptibility to infection (Malamud et al. 2011). While saliva has demonstrated antiviral activity against certain viruses, including Zika and influenza (Conzelmann et al. 2020), the role of specific salivary peptides in SARS-CoV-2 inhibition remains unclear. Notably, the activity of saliva has not been shown to prevent infection by SARS-CoV-2_{WT} or the Omicron and Gamma variants (Conzelmann et al. 2020).

Although bioinformatic approaches such as molecular docking and molecular dynamics have been useful in identifying antiviral compounds (Peele et al. 2020), prior studies have not conducted a validated and comprehensive screening of endogenous salivary peptides. Our study addresses this gap by integrating a systematic bioinformatics pipeline with in vitro validation, demonstrating that naturally occurring salivary peptides can effectively inhibit SARS-CoV-2 infection, including its variants.

We hypothesize that synthetic mimotopes derived from naturally occurring salivary peptides with high binding activity with the RBD motif may effectively prevent SARS-CoV-2 entry into host cells, offering a potential antiviral strategy. Hence, this study aimed to identify salivary peptide–based mimotopes with strong interaction energy with SARS-CoV-2 spike protein and to validate their antiviral activity targeting viral entry blockage.

Materials and Methods

Salivary Peptides, Peptide Homology, Molecular Docking, and Molecular Dynamics

Based on 2,193 salivary peptides (Caseiro et al. 2013), we used an in-house developed pipeline for modeling and docking 298 sequences. This pipeline uses the BLASTp algorithm (Altschul 1997) to filter homologous reference crystallographic 3D protein structures from the Protein Data Bank (Berman et al. 2000) and then perform docking of salivary peptides with SARS-CoV-2 Spike RBD using the HPEPDOCK Server (Zhou et al. 2018). The 4 best affinity energies between peptide-SARS-CoV-2 Spike-RBD complexes from Wuhan (PDB ID 6LZG), Gamma (PDB ID 7EKC), Delta (PDB ID 7WBQ), and Omicron (PDB ID 7WHH) variants were manually selected and submitted to a molecular dynamics (MD) simulation of stability of 100 nanoseconds using GROMACS 2020.3 software (Berendsen et al. 1995) (sequences of Spike RBD used for the docking experiments can be found in the appendix). Stable peptides validated by MD calculations were selected for in vitro experiments (Appendix Table 1, Appendix Fig. 1, Appendix Fig. 2). In addition, to determine potential interference with viral entry, a molecular docking analysis was performed to evaluate the binding affinity of the 4 highest-affinity salivary peptide–SARS-CoV-2 Spike-RBD complexes for their interaction with the host cell ACE2 receptor.

Cell Viability

Cell viability was measured using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; Sigma-Aldrich) method and calculated using the (T/C) × 100% equation (Kamiloglu et al. 2020; Grosche et al. 2023). Cell culture methods are described in the appendix.

Antiviral Assays with VSV-eGFP-SARS-CoV-2-S (VSV-S) and SARS-CoV-2_{WT}

A pseudotyped vesicular stomatitis virus (VSV)–expressing enhanced green fluorescent protein (eGFP) as a marker of infection, in which the glycoprotein gene (G) was replaced by the spike protein of SARS-CoV-2 (VSV-eGFP-SARS-CoV-2-S; GenBank MN908947.3), was used for trial infection assays (Case et al. 2020). VSV-S and peptides at the determined concentration were incubated for 1 h before the naïve Vero E6 cells were infected. After 2 h, the inoculum was removed, and cells were washed with phosphate-buffered saline and filled with DMEM 2%. At 24 h postinfection (h.p.i.), assays were analyzed via fluorescence microscopy, and foci of infection were counted. Antiviral activity was calculated using the (T/C) × 100% equation (Grosche et al. 2023).

The SalivaPep1, SalivaPep2, SalivaPep3, and SalivaPep4 concentrations used in the antiviral assays were selected based on standard protocols to select the highest noncytotoxic concentration (>95% cell viability) with SARS-CoV-2 antiviral activity of each salivary peptide. Vero cells were infected with VSV-eGFP-SARS-CoV-2-S at a multiplicity of infection of 0.005 and simultaneously treated with a 2-fold serial dilution of each compound, ranging from 500 µg/mL to 0.24 µg/mL. Cell viability was assessed using an MTT assay as previously described. Based on these data, the highest noncytotoxic concentration (>95% cell viability) with antiviral activity (Grosche et al. 2023) of 50 µg/mL was determined for SalivaPep1, SalivaPep2, SalivaPep3, and SalivaPep4, and it was used in subsequent experimental steps.

Antiviral assays with SARS-CoV-2 Wuhan-Hu-1, wild-type ($_{WT}$), were conducted in 3 scenarios: (1) general infection assay, in which Vero E6 cells were infected in the presence or absence of selected salivary peptides for 48 h; (2) pretreatment assay, in which cells were treated with peptides for 1 h before infection; and (3) virucidal assay, in which the virus and peptides were incubated together before infection. The infection rates were determined at 48 h.p.i. by measuring cell death due to the infection using a cellular viability assay (Song et al. 2014).

Antiviral Assays with SARS-CoV-2_{WT} and Variants Gamma and Omicron Measured by RT-qPCR

Viral RNA was extracted using the Trizol protocol, and complementary DNA (cDNA) synthesis was performed using the High-Capacity cDNA Archive® kit (Applied Biosystems). The RT-qPCR reactions were performed using a TaqMan Universal PCR master mix kit (Thermo Fisher Scientific). The reactions consisted of each primer and of specific probe to viral nucleocapsid (N) gene (2019-nCoV_N1-F: 5′-GAC CCC AAA ATC AGC GAA AT-3′; 2019-nCoV_N1-R: 5′-TCT GGT TAC TGC CAG TTG AAT CTG-3′; and 2019-nCoV_N1-P: 5′-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3′) (Integrated DNA Technologies), along with 2× Taqman Universal master mix (Thermo Fisher Scientific) and cDNA in DEPEC ultrapure water. All amplifications were conducted on QuantStudio 12 K Flex instrument (Applied Biosystems). Serial 10-fold dilutions of the standard plasmid of SARS-CoV-2 (2019-nCoV_N_Positive Control, 2×10^5 genome copies/µL, obtained from IDT (Integrated DNA Technologies), were used to produce standard curves to quantify the SARS-CoV-2 RNA copies. The limit of detection parameters were similar to SARS-CoV-2_{WT} and the variants. Every RT-qPCR assay was performed in triplicate and included negative (nuclease-free water) and positive controls (Grosche et al. 2023).

Statistical Analysis

Individual experiments were performed in triplicate, and all assays were performed a minimum of 3 times to confirm the reproducibility of the results. GraphPad Prism 8 software was used to assess the statistical differences in the means of readings using the ordinary 1-Way analysis of variance test in multiple comparisons. P values < 0.05 were statistically significant.

Results

Peptides Bind to Spike-RBD Using Molecular Docking

A total of 2,193 protein structures were filtered using a pipeline for modeling and docking sequences with BLASTp and performed molecular docking, then selected and simulated stable peptide-SARS-CoV-2 Spike-RBD complexes for in vitro experiments. HPEPDOCK enabled ranking peptides based on their interaction with SARS-CoV-2's spike protein, identifying SalivaPep1 - PGPRYQPFRLT, SalivaPep2 - GRFGYGYGPY, SalivaPep3 - PAAAPPTLPPYFMKGSIIQLANGELKKV, and SalivaPep4 - RIGRFGYGYGPY as top candidates (Appendix Table 1, Appendix Figs. 1 and 2) for their high scores and MD stability. Peptides SalivaPep2 and SalivaPep4 are derived from Statherin – positions 31 to 40 and 29 to 40, respectively, while SalivaPep3 and SalivaPep1 are derived from Ataxin-1 and Period circadian protein 1 – positions 563 to 590 and 309 to 319, respectively. These salivary peptide interactions with the SARS-CoV-2 spike RBD protein are represented in 3D (Fig. 1A–D), and protein–peptide interactions are detailed as 2-dimensional interaction maps (Fig. 1E–H) to illustrate that SalivaPep1 formed 9 hydrogen bonds, follow by SalivaPep3 and 4 with 4, whereas SalivaPep2 presented only 1 hydrogen bond with the RBD region. On the other hand, all peptides formed electrostatic interactions with the protein receptor, which is crucial for their stability inside the RBD.

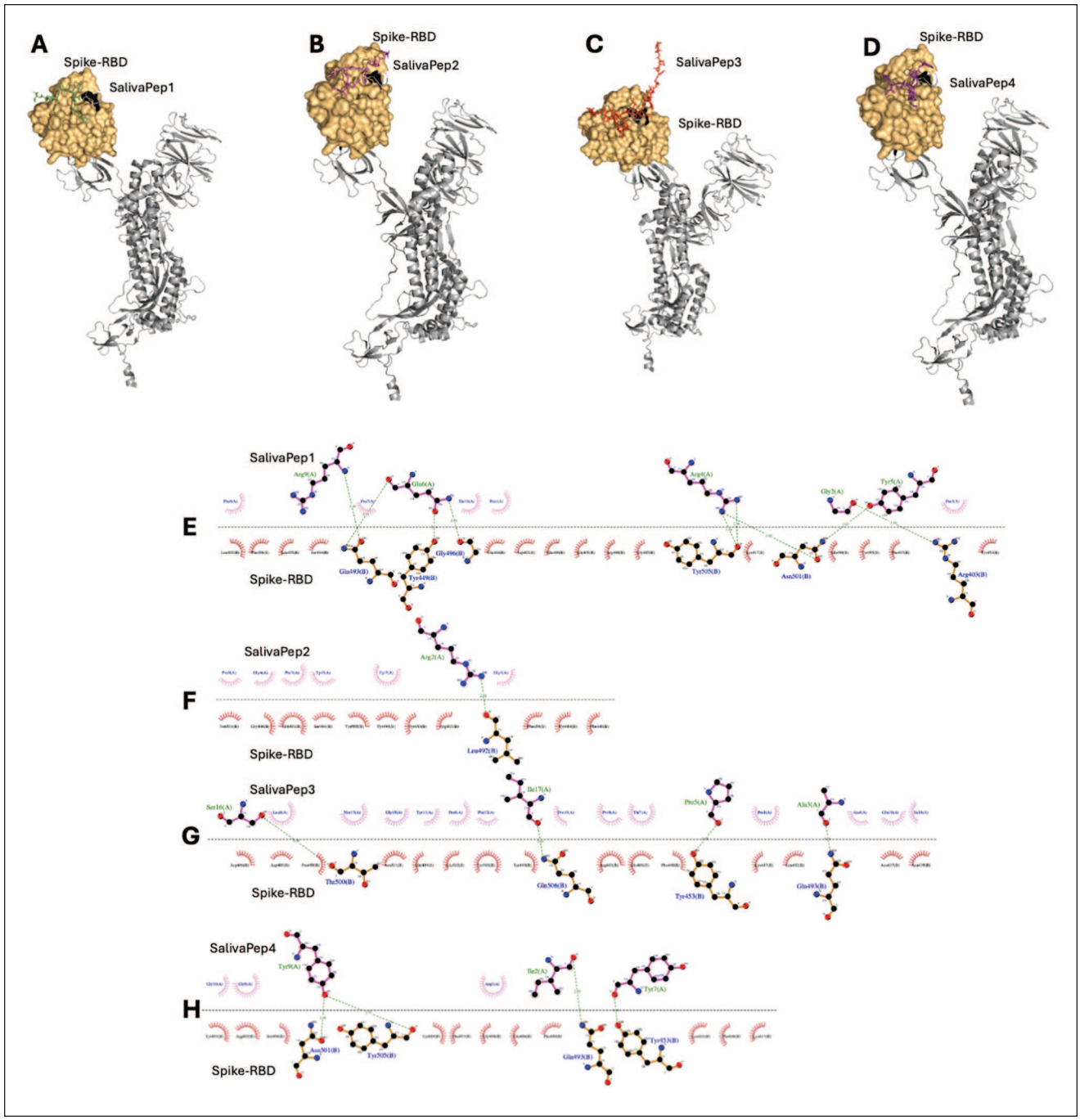

Figure 1. Three-dimensional docking representations for selected salivary peptides interacting with SARS-CoV-2 Spike-RBD. The Spike protein is represented in gray and receptor-binding domain (RBD) in gold. The molecular interaction is represented as (**A**) for SalivaPep1 in green sticks; (**B**) SalivaPep2 in pink sticks; (**C**) SalivaPep3 in red sticks; (**D**) and SalivaPep4 in purple sticks. Two-dimensional interaction maps for SalivaPep1 (**E**), SalivaPep2 (**F**), SalivaPep3 (**G**), and SalivaPep4 (**H**), which interact with SARS-CoV-2 Spike-RBD. Above the dotted lines are the peptides and below them is the Spike-RBD complex. Hydrogen bonds are presented as green dotted lines between the peptides and the Spike-RBD complex.

Table 1 analyzes the docking affinity energies of 4 selected salivary peptides with the SARS-CoV-2 spike RBD of Gamma, Delta, and Omicron variants. The interaction was similar across all variants, with less than 10% energy fluctuation for Omicron compared with Wuhan. Delta and Omicron showed more than 10% variation on average. Peptide SalivaPep3 had higher interaction energy with Wuhan and Gamma, while SalivaPep4 performed best with Delta and Omicron. Compared with Wuhan, SalivaPep3, and SalivaPep4 adapted better to Gamma, but SalivaPep4 had the slightest change with Delta. In

Table 1. Affinity Energy of Selected Peptides and SARS-CoV-2 S Protein Based on a Silico Bioinformatic Molecular Docking Assay.

	Variant						
	Wuhan	Gamma		Delta		Omicron	
Salivary Peptide	Kcal/Mol	Kcal/Mol	%[a]	Kcal/Mol	%[a]	Kcal/Mol	%[a]
SalivaPep1	–218.227	–213.746	–2.1	–194.251	–12.3	–216.522	–0.8
SalivaPep2	–209.840	–214.670	2.2	–192.561	–9.0	–218.531	4.0
SalivaPep3	–227.293	–272.072	16.5	–216.101	–5.2	–213.107	–6.7
SalivaPep4	–218.979	–257.878	15.1	–218.512	–0.2	–227.982	3.9

[a]Percentage of variation when compared with the Wuhan variant.

Table 2. Effect of Salivary Peptides on VERO Cell Viability and VSV-eGFP-SARS-CoV-2 Infectivity.

Salivary Peptide	Concentration (µg/mL)	Cell Viability (%)	Inhibition of VSV Infection (%)
SalivaPep1	50	100.2	44.2
SalivaPep2	50	108.5	37.6
SalivaPep3	50	95.1	67.5
SalivaPep4	50	101.6	49.8

this context, computational simulations revealed that the peptide–RBD complexes failed to establish interactions with ACE2, suggesting a potential inhibitory effect on viral-host recognition. Consequently, these peptides were synthesized for further in vitro testing (Appendix Figs. 3 and 4).

Salivary Peptides Inhibit VSV-eGFP-SARS-Cov-2 Infection

The cell viability for the selected salivary peptides was greater than 95%. Salivary peptide SalivaPep3 showed a higher infection inhibition rate at 67.5%. The infection inhibition rate for salivary peptides SalivaPep1 and SalivaPep4 ranged from 44.2% to 49.8%, respectively. In addition, the salivary peptide SalivaPep2 provided 37.6% of infection inhibition (Table 2).

Despite its strong docking scores and favorable viability data, SalivaPep4 was excluded from further analysis due to its lower synthesis yield compared with SalivaPep2, which offered a more favorable peptide yield despite sharing a similar sequence.

Salivary Peptide Antiviral Assays Using SARS-CoV-2$_{WT}$

In the general infection assay, peptides SalivaPep1 and SalivaPep2 inhibited 32.5% and 31.0% of cell death caused by SARS-CoV-2 infection, respectively. SalivaPep3 inhibited 21.5% of cell deaths caused by viral infection. In the pretreatment assay, SalivaPep3 performed best, inhibiting 34.6% of infection, followed by SalivaPep1 (25.3%) and SalivaPep2 (22.2%). In the virucidal assay, SalivaPep2 had the best antiviral effect, inhibiting 28.4% of infection, followed by SalivaPep3 (24.7%) and SalivaPep1 (21.0%) (Fig. 2). The antiviral properties of lyophilized saliva from healthy individuals were also evaluated for its infection inhibition capabilities; however, no significant inhibitory activity against SARS-CoV-2 was observed (Appendix Fig. 5).

Salivary Peptides Inhibit Infection by Omicron and Gamma Variants

Considering that Omicron and its subvariants have ranked currently as the predominant SARS-CoV-2 strains worldwide and the elevated likelihoods of hospitalization/admission to the intensive care unit among Gamma cases in Brazil, the antiviral effect of salivary peptides was evaluated by titrating viral yields in the presence or absence of treatment using RT-qPCR. On the Gamma variant, SalivaPep1, SalivaPep2, and SalivaPep3 showed up to 74.9% reduction in RNA viral yields compared with the infected control (Fig. 3A). Finally, for the Omicron variant, the reduction in the RNA viral yields ranged from 40% to 50% on the evaluated peptides compared with the infected control (Fig. 3B).

Discussion

Human saliva contains thousands of peptides with the potential for prophylactic and therapeutic applications, targeting a broad range of oral and systemic diseases (Caseiro et al. 2013). While saliva exhibits inherent antiviral defenses against viruses such as Zika and influenza, it failed to inhibit SARS-CoV-2 (Conzelmann et al. 2020), requiring the development of innovative measures, such as virus-binding gum and virucidal oral rinses formulated with specific bioactive molecules. We evaluated lyophilized saliva from healthy individuals for antiviral properties, but no significant inhibitory activity against SARS-CoV-2 was observed. Although lyophilization is expected to preserve the composition of salivary peptides, the lack of antiviral activity is likely due to the concentration and bioavailability of active peptides (de Jong et al. 2011). We demonstrated that naturally occurring salivary peptides present a promising approach against SARS-CoV-2, reducing viral infection cell death and replication by up to 34.6% and 74.92%, respectively. These findings show that endogenously produced salivary peptides effectively interact with the SARS-CoV-2 spike protein, offering a viable strategy to inhibit viral entry and prevent infection without cellular toxicity. Other molecules derived

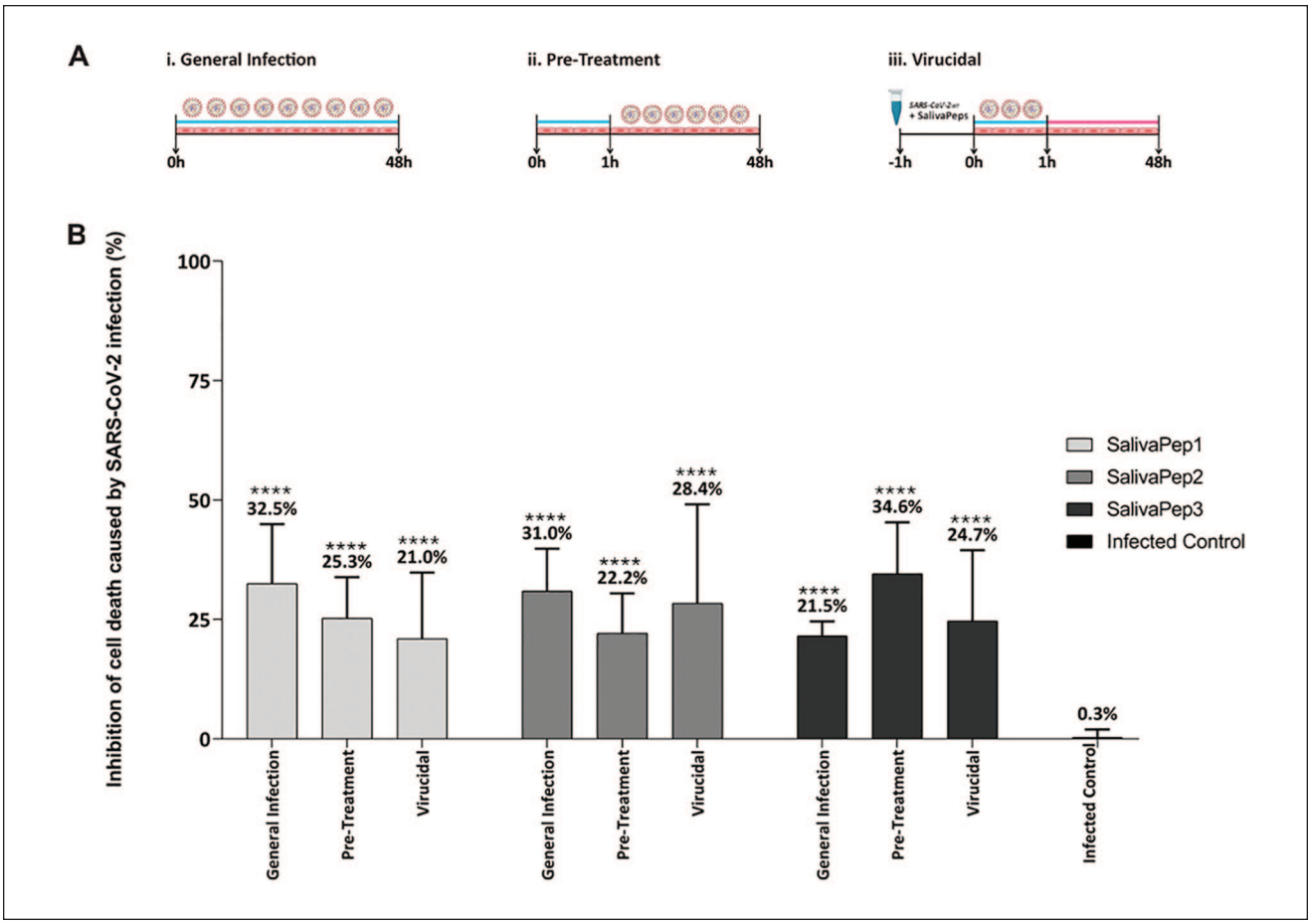

Figure 2. (**A**) Schematic representation of the performed antiviral assays. Vero cells were infected with SARS-CoV-2_{WT} in the following scenarios: (i) general infection assay, where Vero E6 cells were infected in the presence or absence of peptides for 48 h; (ii) pretreatment assay, where cells were treated with peptides for 1 h before infection; and (iii) virucidal assay where the virus and peptides were incubated together before infection. (**B**) Effect of isolated salivary peptides SalivaPep1, SalivaPep2, and SalivaPep3 in inhibiting cell death caused by SARS-CoV-2_{WT} infection. The number of RNA viral yields at 0,01 multiplicity of infection (MOI) on SARS-CoV-2_{WT} Wuhan-Hu-1. A 1-way analysis of variance was performed to compare the effect of each peptide on SARS-CoV-2 Wuhan-Hu-1 strain infection. The mean values of 3 independent experiments, each measured in triplicate, including the standard deviations, are shown. *P* values < 0.05 were considered significant. ****$P < 0.0001$.

from human recombinant soluble ACE2 (hrsACE2) were also effective in reducing viral replication with low toxicity (Monteil et al. 2020). Collectively, our findings support the emerging role of saliva-based therapies in viral infection management, particularly against SARS-CoV-2, highlighting our pioneering efforts in demonstrating their antiviral activity.

Synthetic peptides showed antiviral activity against herpes simplex virus (Sala et al. 2018) and broad-spectrum activity (Brice and Diamond 2020). However, to our knowledge, this is a pioneering study assessing the antiviral activity of endogenously occurring salivary peptides against SARS-CoV-2. These naturally derived salivary peptides offer the dual benefits of inherent low cytotoxicity and high specificity, positioning them as a novel strategy for antiviral therapy of SARS-CoV-2 (Vilas Boas et al. 2019).

Bioinformatics studies underscore the potential of peptide inhibitors in altering spike conformation to inhibit its binding to ACE2 (Baig et al. 2020; Sadremomtaz et al. 2022). Peptides molded from the spike RBD inhibited viral attachment (Chowdhury et al. 2020); however, salivary peptides remain untested. Here, we reported that salivary peptides may inhibit the interaction between the SARS-CoV-2 spike protein and ACE2 via multiple mechanisms, offering a bioinspired alternative to current antiviral compounds. Given the high cost and time involved in synthesizing large libraries of peptides, computational methods such as molecular docking and dynamics simulations offer a practical method for identifying high-affinity peptide candidates (Mahmud et al. 2021), yet our approach remains innovative considering the investigation of salivary peptides with potential interaction. The formation of hydrogen bonding, essential in antiviral activity (De Clercq 2023), was potentially critical in the binding of our salivary peptides to Spike-RBD.

Molecular docking and dynamics simulations have proven effective in predicting peptide activity against several viruses, including SARS-CoV-2, Zika, and dengue, showing agreement

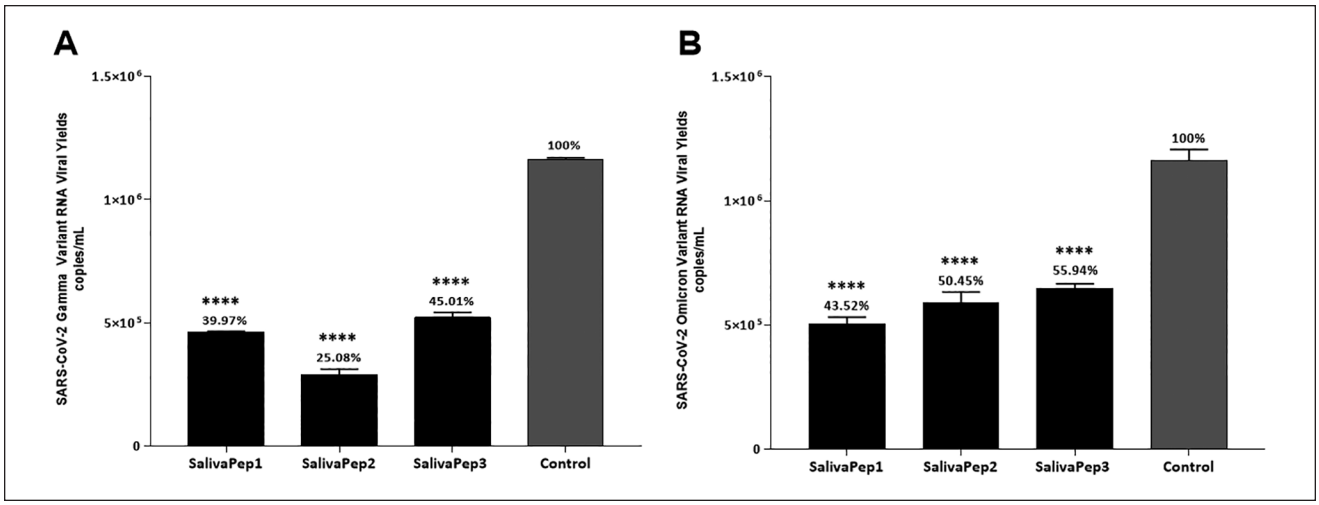

Figure 3. (**A**) Effect of isolated salivary peptides SalivaPep1, SalivaPep2, and SalivaPep3 at 50 µg/mL, and the RNA viral yields at 0,01 multiplicity of infection (MOI) on the SARS-CoV-2_{WT} Gamma variant. (**B**) Effect of isolated salivary peptides SalivaPep1, SalivaPep2, and SalivaPep3 at 50 µg/mL, and the RNA viral yields at 0,01 MOI on the SARS-CoV-2_{WT} Omicron variant.

between computational and experimental results (Chaurasiya et al. 2023). These methods complement each other, enhancing prediction reliability (Biswas et al. 2022). To our knowledge, no prior research has systematically examined salivary peptides against microbial targets through bioinformatics-driven analysis. This strategy enables the on-demand selection of salivary peptides with optimized antimicrobial properties.

Given the high transmissibility and pathogenicity of SARS-CoV-2, we used pseudotyped viruses to minimize infection risks (Grosche et al. 2023). Initial assays used VSV-expressing eGFP and the SARS-CoV-2 Spike protein, allowing us to safely conduct experiments in a biosafety level 2 laboratory (Grosche et al. 2023). Our peptides demonstrated high cell viability and low toxicity, with SalivaPep3 achieving the highest inhibition rate (67.5%), aligning with in silico predictions. SalivaPep2 maintained high viability with low toxicity but showed moderate inhibition, while SalivaPep1 and SalivaPep4 exhibited similar viability and inhibition rates. Notably, these peptides remained effective even at concentrations exceeding natural levels, highlighting their strong potential for prophylactic and therapeutic antiviral applications. Although some treated samples showed a slight increase in cell viability greater than 100%, this variation is minimal and not statistically significant. This result is typical of assays of this nature and does not suggest cellular proliferation or damage. Instead, it highlights that the naturally occurring peptides tested do not induce cellular toxicity and are safe for cellular integrity.

Further assays against the Wuhan-Hu-1 strain confirmed SalivaPep3, SalivaPep1, and SalivaPep2 as effective peptides, showing infection inhibition rates across different scenarios. SalivaPep3 was particularly effective in pretreatment assays, suggesting its potential for prophylactic use. The results indicate that combining these peptides could offer an effective alternative for preventing viral infections. Cyclic peptides targeting SARS-CoV-2's main protease (Curreli et al. 2020) support the potential of peptide-based therapies. However, our study introduces new insights into the application of salivary peptides at various stages of viral replication.

With regard to the antiviral effect of these selected salivary peptides on the RNA viral yields of the Omicron and Gamma variants, we observed reductions of 40% to 74.9%. Other studies have identified broad-spectrum antiviral peptides such as P9, which block viral RNA release by preventing membrane fusion (Zhao et al. 2016), and CPXV012, which inhibits infection through viral envelope interactions. Our findings introduce naturally occurring salivary peptides as novel agents for reducing viral loads in SARS-CoV-2 variants.

Although our study presents limitations, such as potential variability in test conditions and underestimation of viral infectivity, these limitations are inherent to virological research and not exclusive to our work (Tisdale 2000). It is essential to highlight that we implemented rigorous controls and complementary approaches to ensure the robustness and reliability of our findings. Innovative techniques such as viral yield tests are still evolving to address these constraints (Goebel et al. 2016). Physiologically based pharmacokinetic models could aid in translating in vitro results to clinical settings (Honda et al. 2019). Moreover, dosage considerations are crucial for improving in vitro extrapolation accuracy, particularly in the context of SARS-CoV-2 research. Furthermore, while the pseudotyped model does not fully mimic the complex interactions present in a live virus, it is a widely accepted and valuable tool for studying the Spike-RBD/ACE2 interaction under controlled conditions. Pseudotyped viruses were chosen for their safety and controlled conditions, eliminating the need for live SARS-CoV-2 while ensuring reproducibility and minimizing biosafety risks (Case et al. 2020). These assays are well established for studying RBD interactions and evaluating Spike-RBD/ACE2 inhibitors (Grosche et al. 2023). To confirm the robustness of our findings, we validated results using infectious

SARS-CoV-2_{WT}, Gamma, and Omicron variants in a BSL-3 setting, reinforcing the significance of the antiviral activity of salivary peptides.

Various antiviral strategies were explored to reduce SARS-CoV-2 in the oral cavity. Mouth rinses such as povidone–iodine effectively lower salivary viral loads (Carrouel et al. 2021), while oral antivirals such as molnupiravir support infection management (Soriano et al. 2022). Particularly, salivary peptides exhibit broad-spectrum antimicrobial activity and lower toxicity compared with traditional antimicrobial peptides (Vanzolini et al. 2022). To advance their potential, future efforts should optimize peptide design through artificial intelligence–driven refinement, automating high-throughput screening for faster validation and enhancing bioavailability for clinical applications. In addition, integrating genomic surveillance would allow real-time adaptation of peptide sequences to counter emerging viral variants. Afterward, synthetic versions of these peptides will be formulated into oral care products, such as toothpaste, mouthwash, and gels, with rigorous testing in biologically relevant conditions to assess their stability, activity, and therapeutic potential.

Conclusion

The comprehensive analysis of molecular docking energy, detailed interaction maps, and molecular dynamics of salivary peptides SalivaPep1, SalivaPep2, and SalivaPep3 with the SARS-CoV-2 Spike-RBD strongly suggest they could prevent viral replication by effectively inhibiting ACE2 receptor interaction. The robust in vitro performance of these salivary peptides indicates the potential application in oral antiviral drug delivery systems, which can contribute to COVID-19 prevention. In addition, the validated safety profile and robust antiviral efficacy of these salivary peptides underscore their potential as leading candidates for further development into highly selective anti-SARS-CoV-2 agents.

Author Contributions

M.A. Garcia-Júnior, V.R. Grosche, contributed to conception and design, data acquisition, analysis, and interpretation, drafted the manuscript; L.S. Palmeira, C. Teles, G.M. Ferreira, M. Guevara-Vega, R.P.F. Abuna, M.M. Martins, P. Rahal, contributed to data acquisition and analysis, critically revised the manuscript; D.O.S. Martins, M.G. Carneiro, J.S. da Silva, B.S. Andrade, F.R.G. Bergamini, A.C.G. Jardim, R. Sabino-Silva, contributed to conception and design, data acquisition, analysis, and interpretation, critically revised the manuscript; V.A. de Carvalho Azevedo, R.E.F. de Paiva, R.M.P. Vitorino, T.M. Cunha, contributed to data acquisition, analysis, or interpretation, critically revised the manuscript. All authors gave their final approval and agreed to be accountable for all aspects of the work.

Declaration of Conflicting Interests

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