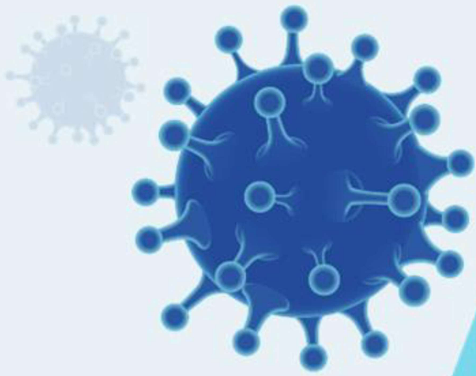
Universidade Federal de Uberlândia

Programa de Pós-Graduação em Ecologia, Conservação e Biodiversidade

EFEITOS ECOTOXICOLÓGICOS E REPRODUTIVOS DO SARS-CoV-2: UM ESTUDO EXPERIMENTAL COM IMPLICAÇÕES PARA A CONSERVAÇÃO DE ESPÉCIES TERRESTRES



Doutoranda: BÁRBARA BEATRIZ DA SILVA NUNES

Orientador: Prof. Dr. Guilherme Malafaia Pinto
Coorientadora: Profa. Dra. Juliana dos Santos Mendonça

Dezembro/2024

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA, CONSERVAÇÃO E BIODIVERSIDADE

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Reuniu-se por videoconferência a Banca Examinadora designada pelo Colegiado do Programa de Pós-graduação em Ecologia, Conservação e Biodiversidade assim composta pelos doutores: Robson José de Oliveira Júnior - UFU; Boscolli Barbosa Pereira - UFU; Maria Betania Melo de Oliveira - UFPE; Bruno da Cruz Pádua - Cefet-MG e Guilherme Malafaia Pinto - IFGoiano, orientador(a) do(a) candidato(a).

Iniciando os trabalhos o(a) presidente da mesa, Dr(a). Guilherme Malafaia Pinto, apresentou a Comissão Examinadora e o(a) candidato(a), agradeceu a presença do público e concedeu ao(à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do(a) Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

A seguir o(a) senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as) que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

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O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

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Dedico este trabalho aos meus pais, que muito me apoiaram e incentivaram; aos meus amigos, pela compreensão nas horas de ausência; e aos meus afilhados, que são um pingo de amor, alegria e esperança nas novas gerações...

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nosso respeito por outros seres vivos, eles respondem com respeito para nós" (ditado Arapaho). Assim, mostro meu respeito e gratidão.

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“Embora ninguém possa voltar atrás e fazer um novo começo, qualquer um pode começar agora e fazer um novo fim”.

Chico Xavier

RESUMO

O SARS-CoV-2, agente etiológico da COVID-19, é um contaminante emergente biológico que repercutiu pandemicamente, impactando a sociedade humana e, principalmente, os mais pobres. Sabe-se que o vírus pode interagir com o ambiente de maneiras ainda não totalmente compreendidas e que tem influenciado negativamente a biologia de diversos organismos aquáticos. Contudo, os possíveis impactos que esse vírus pode ocasionar nos organismos não-alvo foram pouco explorados, limitando o nosso conhecimento sobre suas consequências à biota. Visando determinar os efeitos do SARS-CoV-2 em mamíferos terrestres para antecipar ações de mitigação, avaliou-se os possíveis impactos negativos associados à exposição ao lisado viral sobre a saúde e a capacidade reprodutiva de fêmeas de camundongos C57BL/6J em maturação sexual, e se houve influência na saúde da prole. Para tanto, realizou-se um experimento com duas etapas. A primeira consistiu em expor os animais em fase pré-reprodutiva (pela via oral) ao lisado do SARS-CoV-2 (concentração: 20µg de proteína viral/L) por 30 dias. Posteriormente, avaliou-se os possíveis efeitos da exposição sobre o comportamento das fêmeas (via teste de campo aberto, teste de reconhecimento de objeto e teste de suspensão de cauda) e se houve a indução de efeitos mutagênicos (via teste do micronúcleo em sangue periférico), genotóxicos (via ensaio cometa), hematológicos e bioquímicos (no sangue e em distintos órgãos, a partir de distintos biomarcadores). Não foram evidenciados efeitos hematológicos, mutagênicos ou genotóxicos, porém houve distúrbios comportamentais (hipoatividade, comportamento semelhante à ansiedade e déficit de memória a curto prazo), que foram associados ao estresse oxidativo e ao desequilíbrio dopaminérgico e colinesterásico no cérebro dos animais. Ademais, a elevação dos níveis de bilirrubina e de desidrogenase láctica nestes animais sugere alterações hepáticas, e o desequilíbrio redox, o estresse nitrosativo e a produção elevada de IFN-γ e o infiltrado inflamatório no duodeno, a estrutura folicular perturbada e a presença de vacúolos nas células da granulosa, no ovário, indicam que o grupo exposto ao SARS-CoV-2 apresentou uma toxicidade significativa e com potencial efeito reprodutivo. A segunda fase consistiu em acasalar as fêmeas que sobraram por, no máximo, cinco dias e avaliar dados relativos à gestação, tamanho das ninhadas, biomassa dos filhotes e de biomarcadores de toxicidade. Não foram observadas alterações significativas na duração da gestação, tamanho da ninhada ou biomassa da prole, indicando que os parâmetros reprodutivos não foram afetados. Todavia, a exposição resultou em alterações neurocomportamentais significativas (comportamento semelhante à ansiedade e alterações nos cuidados maternos), associadas a níveis elevados de dopamina e à alteração da atividade da colinesterase em regiões-chave do sistema nervoso central. Adicionalmente, os marcadores de estresse oxidativo e nitrosativo (ROS e NO) estavam elevados nos tecidos mamários e ováricos, sugerindo uma resposta inflamatória persistente. Portanto, este estudo fornece novos conhecimentos sobre a toxicidade das proteínas do lisado do SARS-CoV-2 num organismo terrestre não-alvo da infecção e demonstra que as alterações neuroquímicas e comportamentais observadas podem ter efeitos duradouros. Isto, em ambientes naturais, pode representar um risco significativo para a sobrevivência e estabilidade da população, principalmente através da modulação de vias neuroquímicas e respostas ao estresse em espécies não-alvo.

Palavras-chave: Ecotoxicologia; pequenos mamíferos terrestres; biomarcadores; agentes não-químicos; One Health; Saúde Ambiental.

ABSTRACT

SARS-CoV-2, the etiological agent of COVID-19, is an emerging biological contaminant that has had pandemic repercussions, impacting human society, especially the poorest. It is known that the virus can interact with the environment in ways that are not yet fully understood and that it has negatively influenced the biology of various aquatic organisms. However, the possible impacts that this virus can have on non-target organisms have been little explored, limiting our knowledge of its consequences for biota. To determine the effects of SARS-CoV-2 on terrestrial mammals to anticipate mitigation actions, we evaluated the possible negative impacts associated with exposure to viral lysate on the health and reproductive capacity of sexually maturing female C57BL/6J mice, and whether there was an influence on the health of the offspring. To this end, a two-stage experiment was carried out. The first consisted of exposing pre-reproductive animals (orally) to SARS-CoV-2 lysate (concentration: 20μg viral protein/L) for 30 days. Subsequently, the possible effects of exposure on the behavior of the females were assessed (via the open field test, object recognition test and tail suspension test) and whether there was an induction of mutagenic effects (via the micronucleus test in peripheral blood), genotoxic effects (via the comet assay), hematological and biochemical effects (in the blood and in different organs, using different biomarkers). There were no hematological, mutagenic or genotoxic effects, but there were behavioral disturbances (hypoactivity, anxiety-like behavior and short-term memory deficit), which were associated with oxidative stress and dopaminergic and cholinesterase imbalance in the animals' brains. In addition, the elevation of bilirubin and lactate dehydrogenase levels in these animals suggests liver alterations, and the redox imbalance, nitrosative stress and elevated production of IFN-γ and the inflammatory infiltrate in the duodenum, the disturbed follicular structure and the presence of vacuoles in the granulosa cells in the ovary indicate that the group exposed to SARS-CoV-2 showed significant toxicity with potential reproductive effects. The second phase consisted of mating the remaining females for a maximum of five days and evaluating data on pregnancy, litter size, pup biomass and toxicity biomarkers. No significant changes were observed in gestation length, litter size or offspring biomass, indicating that reproductive parameters were not affected. However, exposure resulted in significant neurobehavioral changes (anxiety-like behavior and changes in maternal care), associated with elevated dopamine levels and altered cholinesterase activity in key regions of the central nervous system. In addition, oxidative and nitrosative stress markers (ROS and NO) were elevated in mammary and ovarian tissues, suggesting a persistent inflammatory response. Therefore, this study provides new insights into the toxicity of SARS-CoV-2 lysate proteins in a terrestrial organism not targeted by the infection and demonstrates that the neurochemical and behavioral changes observed can have long-lasting effects. This, in natural environments, could pose a significant risk to population survival and stability, mainly through the modulation of neurochemical pathways and stress responses in non-target species.

Keywords: Ecotoxicology; small terrestrial mammals; biomarkers; non-chemical agents; One Health; Environmental Health.

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ACh	Acetilcolina
AChE	Acetilcolinesterase
BBI	*Birth Biomass Index* ou Índice de Biomassa ao Nascimento
BChE	Butirilcolinesterase
CA	*Cluster Analysis* ou Análise de Agrupamento
CAT	Catalase
CEs	Contaminantes Emergentes
CEUA	Comissão de Ética no Uso de Animais
CL	*Corpus Luteum* ou corpo lúteo
DSS	Determinantes Sociais da Saúde
ETEs	Estações de Tratamento de Esgoto
FO	*Familiar Object* ou Objeto Familiar
GeoSES	Índice Socioeconômico de Contexto Geográfico para Estudos em Saúde
H&E	*Hematoxylin and Eosin* ou Hematoxilina e Eosina
MDA	Malondialdeído
NOR	*Novel Object Recognition* ou Reconhecimento de Objeto Novo
NO	*Novel Object* ou Objeto Novo
OFT	*Open Field Test* ou Teste de Campo Aberto
OFAI	*Open Field Anxiety Index* ou Índice de Ansiedade em Campo Aberto
OMS	Organização Mundial da Saúde
PCA	*Principal Component Analysis* ou Análise do Componente Principal
ROS	*Reactive Oxygen Species* ou Espécies Reativas de Oxigênio
SPC	Síndrome Pós-COVID
SUS	Sistema Único de Saúde
SOD	Superóxido Dismutase
TNO	Tempo de interação com o Objeto Novo
TFO	Tempo de interação com o Objeto Familiar
TST	*Tail-suspension test* ou Teste de Suspensão de Cauda
WBE	*Wastewater Based Epidemiology* ou Vigilância Baseada em Águas Residuais

SUMÁRIO

1 INTRODUÇÃO

A COVID-19 é uma doença infecciosa de elevada transmissibilidade, cujo agente etiológico foi originalmente relatado em um surto de pneumonia em Wuhan, província de Hubei, China, em 31 de dezembro de 2019 (Huang et al., 2020), sendo caracterizada como pandemia pela Organização Mundial de Saúde (OMS) em 11 de março de 2020. Foi responsável por provocar o colapso dos sistemas de saúde em vários países, o que elevou a quantidade de óbitos (Gonzaga et al., 2020; Lima et al., 2020). O primeiro caso confirmado no Brasil ocorreu na cidade de São Paulo em 26 de fevereiro de 2020, com um paciente que retornara de viagem da Itália (Croda & Garcia, 2020).

O SARS-CoV-2 (família Coronaviridae, gênero *Betacoronavirus*) é o agente etiológico da COVID-19 (Duarte, 2020; WHO, 2020), cujo gênero é composto por vírus que infectam mamíferos (Manzini et al., 2021). O SARS-CoV-2 possui 96% de similaridade genética com o SARS do *Rhinolophus ferrumequinum* (Schreber, 1774) (morcego-ferradura-grande), indicando que também possa ter o morcego como reservatório (Tiwari et al., 2020). No entanto, como a transmissão direta do vírus de morcegos aos humanos dificilmente ocorre, acredita-se que houve um salto para hospedeiros mamíferos intermediários, tal como identificado na SARS [civeta-palmeira-mascarada; *Paguma larvata* (Smith, 1827)] e na MERS [dromedário; *Camelus dromedarius* (Lineu, 1758)] (Baneerjee et al., 2019; Vale et al., 2021), doenças que também são causadas por coronavírus.

As formas mais recorrentes de transmissão do SARS-CoV-2 são por meio do contato direto com uma pessoa infectada (Meyerowitz et al., 2021), inalação de gotículas respiratórias contendo o vírus (Harrison et al., 2020; Phan et al., 2020; Riou & Althaus, 2020) ou pelo acesso a um ambiente contaminado onde partículas suspensas estão presentes (Charlie-Silva et al., 2021; Graham et al., 2020). Além da transmissão clássica, há a possibilidade de contaminação/transmissão fecal-oral ou fecal-nasal (Giacobbo et al., 2021), principalmente por aerossóis de urina, fezes ou toque na base de vasos sanitários (Jayaweera et al., 2020), uma vez que o vírus tem a capacidade de sobreviver em superfícies inanimadas (metal, vidro ou plástico) por até 9 dias (Kampf et al., 2020).

Durante o período de sobrevida do SARS-CoV-2 no ambiente, o vírus e/ou suas partículas virais podem interagir com organismos não-alvo da doença, os quais também podem ser impactados negativamente (Charlie-Silva et al., 2021; Mendonça-Gomes et al., 2021; Gonçalves et al., 2021; Fernandes et al., 2022; Freitas et al., 2022; Gonçalves et al., 2022; Kraus et al., 2022; Luz et al., 2022; Malafaia et al., 2022; Freitas et al., 2023; Luz et al., 2023), servindo de alerta para impactos diretos e indiretos que podem ser ocasionados por vírus e suas partículas biológicas que contaminam o ambiente natural e criam novas condições, constituindo fontes de estresse à vida.

Com a sua disseminação, a COVID-19 teve repercussão de ordem biomédica, epidemiológica, social, econômica, política, cultural e histórica (Gomes et al., 2020; Huang et al., 2020). As várias pesquisas desenvolvidas inicialmente com o SARS-CoV-2 visaram sanar aspectos relacionados à sua disseminação e tratamento da doença, reduzindo ao máximo os óbitos (Bikdeli et al., 2020; Perondi et al., 2021; Sekine et al., 2020; Vizcarra et al.; 2020). Apesar disso, ainda há poucos estudos voltados à elucidação das consequências ambientais e ecológicas dessa pandemia (Charlie-Silva & Malafaia, 2022), a definição do reservatório silvestre do SARS-CoV-2 e a possibilidade de novos transbordamentos zoonóticos.

Sabendo da necessidade de maiores esclarecimentos sobre possíveis consequências à saúde de mamíferos não-humanos e aos ecossistemas advindos da pandemia de COVID-19, almejou-se, neste estudo, investigar os impactos ecotoxicológicos do lisado de SARS-CoV-2 em fêmeas de camundongos C57Bl/6J, modelos experimentais para pequenos mamíferos terrestres. Especificamente, nossos objetivos foram (a) avaliar os possíveis impactos da exposição oral de camundongos fêmeas C57Bl/6J ao lisado viral do SARS-CoV-2, simulando a sua ingestão através de água contaminada; (b) investigar se a ingestão de água contaminada com partículas virais do SARS-CoV-2 no período pré-gestacional pode afetar negativamente a reprodução dessas camundongos fêmeas expostas; e (c) inferir possíveis consequências ecológicas em decorrência da exposição realizada a partir dos dados experimentais.

Inicialmente, partimos da hipótese de que a ingestão da proteína do lisado do SARS-CoV-2 tem impacto na saúde de mamíferos terrestres não humanos, causando alterações comportamentais, hematológicas, serológicas, mutagênicas, genotóxicas, bioquímicas, bem como histopatológicas. Posteriormente, acreditamos que eventuais alterações fisiológicas e histopatológicas observadas no período de maturação sexual podem afetar a capacidade reprodutiva e/ou a qualidade da prole das fêmeas expostas, impactando diretamente no seu *fitness*. Portanto, este estudo procura preencher uma lacuna crucial na compreensão dos impactos indiretos do SARS-CoV-2 em mamíferos não humanos, contribuindo para uma compreensão mais ampla dos riscos ecotoxicológicos associados à pandemia e fornecendo informações valiosas para a gestão ambiental e a preservação da biodiversidade.

Esta tese é constituída pela introdução, por um referencial teórico, dois capítulos experimentais, pelas conclusões gerais e, como apêndice, o material suplementar do primeiro artigo experimental, respectivamente. O segundo capítulo refere-se ao referencial teórico deste trabalho, o qual apresenta uma revisão literária narrativa sobre estudos de diversas áreas do conhecimento afins sobre os impactos globais da pandemia de COVID-19, indo além do que já se sabe sobre a transmissibilidade, patogenia e terapêutica da doença. Nesse capítulo, buscou-se evidenciar o atual conhecimento científico sobre os impactos em recursos hídricos causados pela pandemia de COVID-19, sob a perspectiva da Uma Só Saúde.

O terceiro capítulo é intitulado como "*Beyond the virus: ecotoxicological and reproductive impacts of SARS-CoV-2 lysate protein in C57Bl/6J female mice*". Neste artigo, objetivou-se avaliar os possíveis impactos da exposição oral de camundongos fêmeas da linhagem C57Bl/6J ao lisado proteico do SARS-CoV-2, por meio da simulação de ingestão por água contaminada pelas partículas virais. Este capítulo apresenta os resultados observados na primeira etapa experimental, incluindo os achados comportamentais, genotóxicos, mutagênicos, bioquímicos e histológicos resultantes da exposição direta ao lisado viral. Esse artigo foi aceito para publicação no periódico *Environmental Science and Pollution Research*, ISSN 0944-1344, uma revista de qualis A2, sob o DOI https://doi.org/10.1007/s11356-024-35840-z.

O quarto capítulo é intitulado como "*Pre-mating and gestational trade-offs: reproductive performance, maternal behavior, and organ-specific effects in female mice exposed to SARS-CoV-2 lysate protein*". Nesse capítulo, avaliou-se possíveis impactos reprodutivos das fêmeas expostas decorrentes da exposição ao lisado de SARS-CoV-2 durante seu período pré-reprodutivo. Este corresponde à segunda fase experimental, a qual buscou inferir consequências ambientais e ecológicas da exposição de mamíferos não-alvo ao vírus a partir da análise do estresse oxidativo, neurotoxicidade e comportamentos maternos.

Já o quinto e último capítulo refere-se às considerações finais sobre os resultados observados neste trabalho, além das inferências dessas evidências no âmbito da conservação. Ao final desta tese, apresentou-se o material suplementar do terceiro capítulo como apêndice.

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2 REFERENCIAL TEÓRICO

O Antropoceno tem modificado severamente o meio ambiente, ocasionando pressões que ameaçam os seres vivos, forçando-os a se adaptar rapidamente às novas condições (Tuomainen & Candolin, 2011). A Revolução Industrial transformou profundamente o cenário socioeconômico global ao estimular a urbanização das cidades (Wang et al., 2024), aumentou drasticamente a extração e a utilização de recursos naturais; exacerbou a poluição ambiental a níveis perigosos; afetou as condições climáticas (Power et al., 2018; Ahmed et al., 2021; Wang et al., 2024) e introduziu ruído, luz e vibrações ao ambiente (Mcdonnell & Hahs, 2015). Essa degradação de habitats se tornou um importante problema público internacional, tanto em países desenvolvidos quanto os países em desenvolvimento (Meo et al., 2021), levando a uma crescente extinção de espécies não sinantrópicas. Além disso, as cidades geraram um desequilíbrio entre saúde e doença ao aumentar o risco de interação com novos agentes biológicos e/ou zoonóticos, dada a maior proximidade entre humanos e entre humanos e animais (Esposito et al., 2023).

Com o desenvolvimento tecnológico, houve o desenvolvimento de novas substâncias e microrganismos utilizados nas atividades antrópicas e que, apesar de terem permitido grandes avanços (ex. criação de novos medicamentos), trouxeram riscos à saúde pública e ao meio ambiente ainda não completamente conhecidos (Wang et al., 2024). Sabe-se, por exemplo, que houve uma evolução de patógenos induzida pelo desenvolvimento de novas moléculas químicas (Lebarbenchon et al., 2008) e pela modificação genética de microrganismos (Hanlon & Sewalt, 2021). Os Contaminantes Emergentes (CEs) são produtos químicos ou agentes biológicos que representam uma ameaça percebida, potencial ou real para a saúde humana e/ou ao meio ambiente e para os quais não existem normas sanitárias publicadas, seja por serem provenientes de uma fonte recente ou por terem desenvolvido uma nova via (Picó & Barceló, 2023; Wang et al., 2024). A propagação dessas substâncias pode perturbar vários processos biológicos fundamentais, resultando em respostas fisiológicas, psicológicas/comportamentais desadaptativas às condições ambientais da biota, inclusive de seres humanos (Domingo et al., 2020; Angelier, 2022).

A propagação de CEs pode causar alterações no crescimento, no desenvolvimento (Fernandes et al., 2022; Gonçalves et al., 2022; Malafaia et al., 2022; Tyrkalska et al., 2023) e no comportamento (Tuomainen & Candolin, 2011; Mendonça-Gomes et al., 2021), resultando em declínios dramáticos na abundância e biodiversidade de insetos (Sánchez-Bayo & Wyckhuys, 2019), avifauna (Rosenberg et al., 2019) e espécies aquáticas (Tickner et al., 2020). Ainda, os poluentes ambientais podem interferir em várias características necessárias do processo reprodutivo e alterar a quantidade e qualidade da prole, ou seja, a aptidão de um indivíduo (Orr, 2009), perturbando, assim, o sucesso reprodutivo global de uma população (Aulsebrook et al., 2020). Essas interferências podem ocorrer nos processos de seleção sexual (Tomkins et al., 2018), na proporção de machos/fêmeas (Visser et al., 2009), na comunicação sexual (Shannon et al., 2016), no desenvolvimento sexual, no próprio sistema reprodutor e órgãos associados (Lange et al., 2009; Baron et al., 2017; Cook et al., 2019), no sucesso de fertilizações (Han et al., 2019), quanto por alterações no comportamento parental e cuidado com a prole (Cianchetti-Benedetti et al., 2018). Por exemplo, em mariposas fêmeas adultas [*Helicoverpa armigera* (Hübner, 1805), família Noctuidae], essas substâncias podem interferir nos processos de seleção sexual e sucesso do acasalamento, ao comprometer a produção, transmissão e detecção de sinais importantes na comunicação sexual, como os feromônios, reduzindo, assim, a aquisição de parceiros e o sucesso do acasalamento (Eliyahu et al., 2003). Ainda, poucos estudos se concentraram nos impactos potenciais de agentes tóxicos não químicos (por exemplo, patógenos humanos) em organismos não-humanos (Malafaia et al., 2024), o que evidencia a necessidade de abordagens multidisciplinares e integrais relacionando as interconexões da saúde humana, animal, vegetal e ecossistêmicas.

A Organização Mundial da Saúde (OMS) definiu, em 1978, que a saúde humana é um estado de completo bem-estar físico, social e mental (WHO & UNICEF, 1978), ou seja, não se resume à ausência de enfermidades, mas também considera o cuidado integral das dimensões afetiva, cognitiva, sociocultural, comportamental e ecológica (Antonovsky, 1979). Apesar disso, as nações constantemente negligenciam as questões sociais e ambientais, fato que impede a redução de mortes evitáveis. Desse modo, a OMS reconheceu que a saúde humana, de animais, plantas e ecossistemas são intimamente ligados e interdependentes, estabelecendo o conceito da Uma Só Saúde (Picó & Barceló, 2023). Conforme esse conceito, a interface entre humanos e outros animais (estimação, produção e os silvestres) faz com que estejamos em um relacionamento constante, inclusive pelo compartilhamento dos mais diversos ambientes, constituindo uma fonte de doenças para ambos (Zappulli et al., 2020). Neste sentido, essa abordagem é uma ferramenta promissora para integrar saberes, fornecendo uma visão panorâmica e abrangente sobre a realidade ambiental, embora ainda seja incipiente e apenas tenha começado a se estabelecer em diversos países. No Brasil, por exemplo, a sua implementação apenas se iniciou com a criação do Comitê Técnico Interinstitucional de Uma Só Saúde pelo Decreto nº 12.007 de 25 de abril de 2024, como uma ação multisetorial vinculada ao Sistema Único de Saúde (SUS) (Brasil, 2024), após 16 anos da criação da One Health Comission em 2008 pela OMS (Leifels et al., 2022).

Em decorrência da pandemia de COVID-19, o isolamento social e a interrupção momentânea de muitas atividades foram ações propostas como uma opção adequada para reduzir a velocidade de propagação da doença (Wu et al., 2020) e assegurar o acesso adequado da população ao tratamento (Rafael et al., 2020), as quais alteraram a demanda global por produtos e os resíduos produzidos, modificando temporariamente a pegada ecológica humana. Segundo Picó & Barceló (2023), como pontos positivos da pandemia a nível mundial, houve uma melhoria geral na qualidade do ar, melhoria da qualidade das águas subterrâneas e limpeza de praias, além da redução da poluição sonora. No entanto, a pandemia também desencadeou um aumento no desperdício médico, na incineração, no uso de desinfetantes (desinfetantes domésticos, desinfetantes para mãos, biocidas) e produtos farmacêuticos anti-COVID-19 (antibióticos, antivirais, glicocorticoides, etc), na produção de resíduos plásticos e uma diminuição da qualidade do ar interno (Faridi et al., 2020; Tagorti & Kaya, 2022; Picó & Barceló, 2023).

A alteração da pegada ecológica global pelo contexto pandêmico resultou em novos cenários ambientais e no do surgimento de novas formas de contaminação e propagação de organismos patogênicos, os quais trouxeram consequências diretas e indiretas à fauna. Com as medidas de isolamento social e a grande redução na atividade humana e nos ruídos, houve a modificação do comportamento dos animais, sendo que os domésticos ficaram mais estressados pelo confinamento; enquanto que os sinantrópicos tiveram sua alimentação restringida pela redução da oferta de comida, e os selvagens passaram a se movimentar nas ruas das cidades, mesmo durante o dia (Bar, 2021). Além disso, houveram avistamentos raros de animais se aproximando da costa, nidificando em locais incomuns e permanecendo mais em estuários (Monks, 2020). Ao mesmo tempo, toda a biota passou a sentir os efeitos do aumento da produção e descarte de resíduos farmacêuticos, sanitários e plásticos. Assim, o efeito em cascata no ambiente pela ação antropogênica impacta a biota que, por sua vez, também pode impactar o ser humano e o seu bem-estar, dada a sua interconexão. Como a COVID-19 se tornou uma doença respiratória sazonal recorrente (Murray & Piot, 2021), as mudanças no padrão de contaminação ambiental podem ser muito mais permanentes do que se pensava ao início da pandemia.

Apesar do crescente número de estudos avaliando as consequências diretas e indiretas aos humanos relacionadas à exposição ao SARS-CoV-2 e/ou suas partículas virais, ainda há a necessidade de se compreender melhor se e como a pandemia da COVID-19 pôde afetar a fauna silvestre, além dos efeitos que poderão ser observados em novos surtos dessa doença. Assim, procedemos essa revisão literária narrativa objetivando relacionar estudos de diversas áreas do conhecimento afins sobre os impactos globais da pandemia de COVID-19 sob a perspectiva da

Uma Só Saúde, indo além do que já se sabe sobre a transmissibilidade, patogenia e terapêutica da doença. Neste sentido, analisamos essas interações sob uma perspectiva ecológica, abordando lacunas de conhecimento necessárias de elucidação sobre impactos associados à contaminação do meio aquático por SARS-CoV-2. Espera-se que essa abordagem integrada possa auxiliar na promoção de um ambiente de qualidade, saudável e sustentável a todas as formas de vida, além de oportunizar a antecipação de ações voltadas à redução de seus efeitos no ambiente.

2.1 VULNERABILIDADE SOCIAL, POLUIÇÃO AMBIENTAL E COVID-19

Historicamente, as várias endemias e pandemias têm afetado mais severamente as populações mais pobres e marginalizadas, por serem mais vulneráveis social e economicamente, resultando em condições de vida precárias e na falta de acesso a serviços de saúde, rendimentos e educação adequados (Acharya, 2022). Em todo o planeta, durante a pandemia, as comunidades de baixo rendimento apresentaram taxas de mortalidade mais elevadas, influenciadas por fatores como condições de vida mais densas, empregos que não permitem o trabalho remoto e menor acesso a cuidados de saúde (Hawkins et al., 2020). Ainda, as populações de menor renda geralmente são pressionadas a viver em locais com maiores índices de intercorrências ambientais e, muitas vezes, a morar em áreas impróprias para ocupação, como morros e favelas (White et al., 2021; Hartono et al., 2022). Assim, as questões ambientais pré-existentes (água disponível, luz solar acessível, temperatura adequada, umidade do ar, desastres naturais, eventos de vida estressantes e saneamento básico) do seu local de moradia tornam a população mais pobre mais vulnerável (Tagorti & Kaya, 2022).

Notavelmente, a crise econômica mundial gerada pela pandemia ampliou a proporção de pessoas em pobreza extrema, de pessoas desnutridas (Jackson et al., 2021) e de pessoas mais vulneráveis socialmente, as quais estavam em risco crítico com relação à COVID-19 (Sumner et al., 2020). Além disso, a pandemia foi responsável por um esgotamento significativo da saúde mental humana, uma vez que o isolamento social, o desemprego, o confinamento e a perda de muitos entes queridos promoveram o estresse, a ansiedade, a depressão e o aumento das taxas de suicídio, o que, por sua vez, promoveu uma maior carga financeira e mental para os familiares sobreviventes, além de promover a sua insegurança alimentar (Tagorti & Kaya, 2022). Esse cenário, aliado à desnutrição (deficiência ou excesso de calorias combinado com a falta de pelo menos um nutriente), provavelmente aumentou as tensões psicológicas durante a pandemia (Saunders et al., 2011) e levou a alterações no sistema imunológico, deixando essa população ainda mais suscetível a doenças infecciosas (Spoede et al., 2021).

Nos Estados Unidos, por exemplo, verificou-se que as minorias étnicas e os grupos de baixa renda sofreram desproporcionalmente com taxas de infeção e de mortalidade mais elevadas devido à COVID-19 (Van Dorn et al., 2020). As comunidades pobres são suscetíveis à exposição à poluição porque vivem em locais com menor acesso ao saneamento básico, contribuindo para um maior risco de adoecer gravemente devido a infecções como a COVID-19 (Briggs, 2003). A discrepância entre a qualidade da saúde e a vulnerabilidade social é ainda mais significativa nos países em desenvolvimento. Nestes países, os riscos para a saúde são geralmente mais elevados, pois a sinergia entre a pobreza, uma legislação ambiental fraca ou mal aplicada e a falta de investimento em tecnologias geram níveis mais elevados de poluição (Briggs, 2003). Além disso, as associações entre contaminantes ambientais e saúde são complexas e, muitas vezes, mal caracterizadas, resultando em milhões de mortes prematuras anualmente e em uma degradação ambiental generalizada (Wang et al., 2024).

Uma vez que os Determinantes Sociais da Saúde (DSS) são essenciais para caracterizar e monitorar a situação de saúde de uma população, as políticas públicas de estratégias de enfrentamento e recuperação devem ter em conta as especificidades e necessidades das populações mais vulneráveis para mitigar impactos desproporcionais e promover uma sociedade mais justa e resiliente (Marmot et al., 2008). Nessa perspectiva, Barrozo et al. (2020) propuseram um Índice Socioeconômico de Contexto Geográfico para Estudos em Saúde (GeoSES) para sintetizar informações socioeconômicas relevantes para contextualizar a saúde da população, avaliar e monitorar as desigualdades, desenvolver estratégias de alocação de recursos e serviços, além de possibilitar a criação, implementação e execução de políticas públicas intersetoriais mais eficientes e justas, melhorando a saúde da população como um todo e reduzindo as desigualdades. O GeoSES é avaliado de acordo com a abrangência das unidades federativas (município, estado e União) e está alinhado com os principais índices de saúde da população (educação, mobilidade, pobreza, riqueza, renda, segregação e privação de recursos e serviços), abrangendo 46 variáveis e, assim, pode ser um índice que mostra o risco relativo de mortalidade por causas evitáveis no Brasil (Barrozo et al., 2020).

Embora o GeoSES seja uma ferramenta essencial na gestão da saúde no Brasil, ainda é necessário integrar a *expertise* de vários campos do conhecimento (e.g., medicina, veterinária, ciências ambientais e saúde pública) visando reduzir os riscos de CEs para aumentar o bem-estar de todos os organismos (Wang et al., 2024). Esta necessidade é amplificada pois, em certa medida, a pandemia da COVID-19 pode ser considerada uma consequência indireta das alterações ambientais globais, como a degradação dos solos, a destruição da camada de ozono, a poluição e a urbanização, que criaram alterações significativas no ambiente e na saúde humana (Chakraborty & Maity, 2020). Assim, ao dotar o Estado de ferramentas mais práticas e eficazes, ele poderá atuar de forma mais eficiente no enfrentamento das crises sanitárias, garantindo o direito fundamental de acesso à saúde dos brasileiros, bem como promover a equidade e a justiça social, garantindo a todos os cidadãos condições dignas de vida e acesso universal a serviços essenciais, especialmente em tempos de crise sanitária.

2.2 A VIGILÂNCIA AMBIENTAL E AS DIFERENTES MANIFESTAÇÕES DA COVID-19

Em humanos, a infecção com SARS-CoV-2 pode se apresentar clinicamente em indivíduos assintomáticos, indivíduos com sintomas leves e indivíduos com doença respiratória aguda ou pacientes com pneumonia em diferentes graus de gravidade (Lai et al., 2020). Nos casos sintomáticos, a apresentação clínica relacionava-se principalmente com processos infecciosos no sistema respiratório e digestivo. Segundo Lu et al. (2020), os principais sintomas foram febre (88,3%); tosse (68,6%); mialgia ou fadiga (35,8%); expectoração (23,2%); dispneia (21,9%); cefaleia ou tontura (12,1%); diarreia (4,8%) e vômitos ou náuseas (3,9%). A maioria dos pacientes (86%) progride com um bom prognóstico; 14% necessitam de oxigenoterapia em uma unidade hospitalar e menos de 5% desse grupo necessitam de terapia intensiva (WHO, 2020). As complicações da infecção podem progredir para pneumonia, síndrome respiratória aguda grave (SRAG), lesão cardíaca ou renal, infecção secundária de choque, além de disfunção cardíaca, hepática e renal (Guo et al., 2021; Huang et al., 2020). Usualmente a mortalidade estava relacionada a idosos, comorbidades e casos críticos (Dowd et al., 2020; Wu & Mcgoogan, 2020).

Sabe-se que, com humanos, a Síndrome Pós-COVID (SPC) inclui queixas respiratórias, cardiovasculares, digestivas e neurológicas (Lechner-Scott et al., 2021; Salamanna et al., 2021; Oronsky et al., 2023), principalmente em decorrência da permeabilidade dos tecidos cerebrais ao vírus (Zhang et al., 2020), da hiperestimulação imunológica e da neuroinflamação, mas também pelo estresse psicológico (ex. ansiedade e depressão) (Kempuraj et al., 2020) e pelo efeito sobre a microbiota intestinal (Almeida et al., 2023). Ainda em humanos, sabe-se que o SARS-CoV-2, pode infectar órgãos do sistema reprodutor feminino (ovário, o útero e a vagina) e eixo gonadal hipotálamo-hipófise, levando à infertilidade e a vários distúrbios menstruais (Madaan et al., 2022). Além disso, há o alerta de que os hormônios sexuais femininos (estradiol e progesterona) influenciam o comportamento de mastócitos, podendo levar a potenciais distúrbios relacionados à função reprodutiva feminina (distúrbios hormonais, evolução da endometriose, fertilidade feminina e evolução da gravidez), embora tal relação ainda não tenha sido comprovada como condição pós-COVID-19 (Szukiewicz et al., 2022).

Vários animais são suscetíveis aos coronavírus, os quais podem afetar o seu trato respiratório (bovinos, canídeos, galináceos, mamíferos marinhos, murinos, mustelídeos, primatas não humanos, ruminantes selvagens e suínos), o intestino delgado (bovinos, canídeos, caprinos, cervídeos, equinos, felinos domésticos e selvagens, galináceos, guaxinim, leões, leporídeos, murinos, mustelídeos, ovinos, suínos), o intestino grosso (bovinos e ruminantes selvagens), causar lesões neurais (felinos domésticos e selvagens, furões e camundongos), vasculite (mustelídeos e felinos selvagens), além de ocorrer secundariamente nos rins, fígado, linfonodos, pâncreas, baço, timo, olhos e coração (Abdel-Moneim & Abdelwhab, 2020; Zappulli et al., 2020).

Com relação ao SARS-CoV-2, acredita-se que o vírus possua certa plasticidade para infectar hospedeiros, embora esteja primariamente relacionado à infecção de humanos. Isso, pois, os vírus evoluem rapidamente devido à sua alta capacidade de mutação, seja por incorporações errôneas, inserções, deleções ou recombinação, aliada à seleção natural para características favoráveis, como replicação viral mais eficiente, transmissão e evasão das defesas do hospedeiro, o que pode lhe conferir uma maior virulência (McCormick et al., 2021), mas, também, lhe permitir infectar outros organismos. Inicialmente, por exemplo, acredita-se que houve um salto inicial de uma linhagem viral ancestral que circulou em morcegos para humanos, possivelmente pelo contato direto com o hospedeiro infectado, seja pelo seu manuseio ou a sua ingestão (Tan et al., 2022). Ainda segundo esses autores, após a ampla disseminação entre humanos e pela relação próxima de humanos com outros animais, a COVID-19 atualmente tem atuado como uma zoonose reversa, com transmissão significativa para mamíferos domésticos e selvagens que, consequentemente, se tornaram reservatórios da doença (Figura 1).

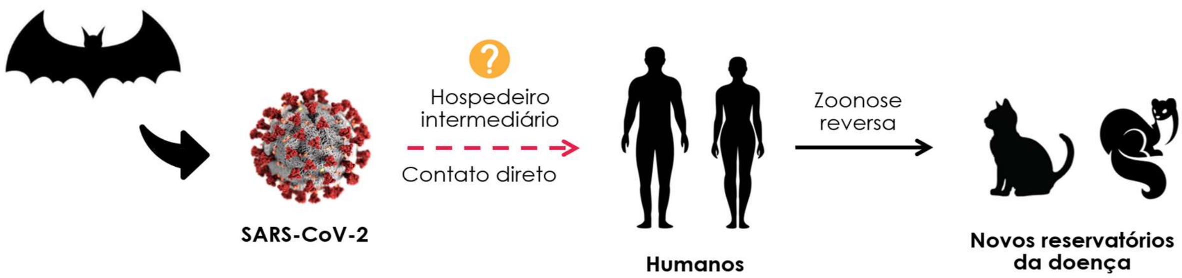

Figura 1. Resumo esquemático da trajetória de evolução do vírus SARS-CoV-2, desde sua origem de uma linhagem viral ancestral circulante em morcegos até o transbordamento zoonótico de humanos para outros animais que se tornaram novos reservatórios da doença.

Comprovou-se que alguns animais domésticos foram impactados negativamente pela presença do vírus. O estudo conduzido por Shi et al. (2020), por exemplo, consistiu na inoculação intranasal com o SARS-CoV-2 (10^5 unidades formadoras de placas (PFU) de F13-E ou CTan-H) em furões [*Mustela putorius furo* (Linnaeus, 1758), família Mustelidae], gatos [*Felis catus* (Linnaeus, 1758), família Felidae], em cães Beagle [*Canis lupus familiaris* (Linnaeus, 1758), família Canidae] e em porcos [*Sus scrofa domesticus* (Linnaeus, 1758), família Suidae]. Observou-se que, em furões, houve a replicação viral no trato respiratório superior e a presença do RNA viral no trato intestinal por até 8 dias, sem causar doença grave ou morte; em gatos houve a infecção do seu trato respiratório, mas não no intestino delgado; em cães, houve baixa infecção viral, indicando que os cães têm baixa suscetibilidade ao SARS-CoV-2; enquanto que porcos não foram suscetíveis (Shi et al., 2020). Ainda, provou-se que o vírus pôde ser cultivado a partir de pulmões de furões (Zappulli et al., 2020). Ainda, estes estudos evidenciaram que a exposição de animais ao vírus não induz um quadro infeccioso semelhante ao observado em humanos, porém impacta outros organismos expostos de diversos modos, os quais são influenciados pela sua classificação biológica e proximidade taxonômica aos humanos, além do método de exposição utilizado.

Após algum tempo no ambiente, há a desnaturação e lise do vírus, com a consequente liberação de partículas virais e do RNA do SARS-CoV-2 no meio. Apesar dos crescentes estudos sobre este processo, as suas consequências ao ambiente não são totalmente conhecidas (Picó & Barceló, 2023). Organismos aquáticos e semiaquáticos, por exemplo, têm sua saúde afetada pelos fragmentos peptídicos do SARS-CoV-2, levando a danos aos indivíduos com potencial de impactar suas populações naturais (Fernandes et al., 2022; Gonçalves et al., 2022, Malafaia et al., 2022; Luz et al., 2023). Evidenciou-se que, em espécimes de guppy [*Poecilia reticulata* (Peters, 1859), família Poeciliidae], a exposição às partículas virais do SARS-CoV-2 alterou o seu comportamento, induziu desequilíbrio redox, afetou o seu crescimento e desenvolvimento (Malafaia et al., 2022), além de induzir instabilidade genômica e danos ao DNA (Gonçalves et al., 2022). Luz et al. (2023) demonstraram que a exposição de *zebrafish* adulto [*Danio rerio* (F. Hamilton, 1822), família Cyprinidae) induz efeito mutagênico, desequilíbrio redox, alterações nos comportamentos de memória de habituação e de resposta antipredatória em cardumes, além de estresse nitrosativo e resposta imune específica. Ainda, Charlie-Silva et al. (2021) demonstraram que a exposição curta (24 h) de girinos a esses peptídeos foi suficiente para afetar a sua saúde e a sua memória, uma vez que houve o aumento de vários biomarcadores preditivos de estresse oxidativo e a alteração na atividade da acetilcolinesterase (AChE). Esses efeitos observados evidenciam que a exposição desses organismos ao SARS-CoV-2 ou aos seus peptídeos virais pode torná-los mais suscetíveis à predação por não reconhecer possíveis predadores e pelo impacto direto em seu vigor e desempenho físico. Desse modo, essa exposição pode afetar as suas interações sociais, reprodução e dinâmicas populacionais em ambientes naturais e, por conseguinte, pode influenciar cadeias tróficas e os ecossistemas aquáticos como um todo.

Apesar de camundongos não serem naturalmente suscetíveis ao SARS-CoV-2 por não possuírem receptores ACE2 (Mullick et al., 2020), há a evidência de que camundongos machos (Swiss e C57Bl/6J), após 24 h de administração intraperitoneal de fragmentos peptídicos do SARS-CoV-2 (580 µg/kg), apresentaram déficit de memória associada à supressão da atividade cerebral da acetilcolinesterase, menor atividade da superóxido dismutase e da catalase no cérebro, evidenciando maior sensibilidade dos camundongos C57Bl/6J aos peptídeos virais (Luz et al., 2022). Além disso, há evidências que a infusão de proteína spike no cérebro de camundongos induz disfunção cognitiva tardia, microgliose hipocampal tardia e perda de sinapses, indicando que o vírus é capaz de induzir efeitos tardios (“COVID tardia”) também em camundongos (Fontes-Dantas et al., 2023).

Com relação à vigilância ambiental para o SARS-CoV-2, gatos e furões são suscetíveis ao vírus, o replicam eficientemente, além de o transmitir a outros indivíduos da mesma espécie (Halfmann et al., 2020; Richard et al., 2020). Ainda, Xiao K. et al. (2020) indicou que 68% dos pangolins selvagens na China e Malásia foram infectados com o Pangolin-CoV, vírus semelhante ao SARS-CoV-2. Este vírus, por sua vez, pode se tornar uma ameaça à saúde pública se o comércio de vida selvagem não for controlado de forma eficaz (Huang et al., 2024). Assim, a vigilância do vírus nessas espécies deve ser considerada como um complemento à eliminação da COVID-19 em humanos (Richard et al., 2020; Shi et al., 2020) e como medida de controle ambiental da COVID-19 e de doenças similares que possam surgir.

Considerando essas questões, a existência da possibilidade de transmissão zoonótica da COVID-19 evidencia a necessidade de avaliações mais abrangentes, esclarecendo os impactos ambientais e em ecossistemas causados por essa doença, sobre a possibilidade de novas interações homem-natureza que possam contribuir para a manutenção/infecção do vírus no ambiente (ex. outras espécies suscetíveis e reservatórios de infecção) ou novos transbordamentos zoonóticos, como, por exemplo, efeitos indiretos de fatores ambientais por mudanças climáticas. Ainda, é mister a compreensão holística das interações entre fatores humanos, animais e ambientais para desenvolver estratégias abrangentes que abordem as rotas de transmissão entre espécies, melhorando as saúdes humana e animal (Sellars et al., 2021). Para tanto, estudos recentes têm sugerido a utilização da metagenômica e da bioinformática como ferramentas auxiliares à detecção precoce de novos surtos (Wani et al., 2024), embora sejam métodos limitados pelo seu alto custo.

2.3 SANEAMENTO BÁSICO E ECOTOXICIDADE DO SARS-CoV-2

Embora a transmissão clássica da COVID-19 seja a mais usual e significativa, diversas pesquisas alertam para a possibilidade de infecção também pelo contato direto com esgoto doméstico ou água contaminada (Jones et al., 2020; Polo et al., 2020; Xiao, F. et al., 2020b; Albastaki et al., 2021; Charlie-Silva et al., 2021; Elsamadony et al., 2021; Gonçalves et al., 2021; Pandey et al., 2021; Wu et al., 2022), com os aerossóis de urina ou fezes gerados nos sistemas de bombeamento e tratamento de efluentes (Gormley et al., 2020; Jayaweera et al., 2020; Usman et al., 2021) e via conexões defeituosas de ralos de piso interligados com a tubulação principal de edifícios/casas (Shi et al., 2021; Charlie-Silva & Malafaia, 2022). Estudos relatam a presença do SARS-CoV-2 em fezes e *swabs* anais de pacientes infectados pela COVID-19, mesmo se fossem assintomáticos ou já possuíssem alta clínica (Xiao, F. et al., 2020a, 2020b; Xing et al., 2020), com o RNA viral permanecendo detectável por até 33 dias após os sintomas iniciais (Wu et al., 2020; Xing et al., 2020). Além disso, foi identificada a presença de SARS-CoV-2 na urina de humanos infectados (Tampe et al., 2021) e que o material genético do coronavírus presente no esgoto aumentou linearmente com o aumento de casos confirmados de COVID-19 (Kumar et al., 2020).

Nas primeiras duas grandes epidemias de Cov registradas [SARS (2002-2003) e MERS (2012-2015)], práticas inadequadas de descarte de dejetos humanos causaram surtos e eventos de superdifusão (Gormley et al., 2017; Huang et al., 2024). Assim, a carga viral presente nas excretas humanas pode se difundir em recursos hídricos, levando à transmissão fecal-oral para novos hospedeiros (Panchal et al., 2021). Durante os surtos de COVID-19, o SARS-CoV-2 foi detectado em efluentes de vários países dos continentes americano, europeu e asiático, contribuindo para a sua transmissão secundária (Liu et al., 2021), principalmente em locais em que se observou um grande número de casos/mortes e em que o saneamento básico é inadequado (Aguiar-Oliveira et al., 2020; Guerrero-Latorre et al., 2020; Rimoldi et al., 2020; Mahlknecht et al., 2021). Neste sentido, a estratégia *Wastewater Based Epidemiology* (WBE) – ou Vigilância Baseada em Águas Residuais – foi uma ferramenta útil para avaliar o vírus em águas residuais, sendo uma importante estratégia para rastrear a propagação da transmissão da COVID-19 (Graham et al., 2020), informar sobre mutações do SARS-CoV-2 (Wu et al., 2020; Charlie-Silva et al., 2021) e alertar precocemente sobre novos surtos da doença. Tandukar et al. (2024) apenas fez uma ressalva com relação à aplicabilidade da WBE em países de baixa e média renda, nos quais esta ferramenta precisaria ser adaptada para considerar matrizes de amostragem alternativas, já que nesses países as Estações de Tratamento de Esgoto (ETEs) são frequentemente inadequadas, mal conservadas, sobrecarregadas e ineficientes, resultando na descarga direta de águas residuais em corpos hídricos.

Outro fator essencial para avaliar os riscos à saúde associados à água contaminada é saber por quanto tempo o SARS-CoV-2 ou suas partículas virais podem permanecer em ambientes aquosos. Conforme demonstrado na Tabela 1, as taxas de ocorrência, decaimento e persistência do SARS-CoV-2 infeccioso viável e do RNA viral nas diferentes matrizes de água dependem principalmente da temperatura, permanecendo por um tempo mais prolongado em temperaturas mais baixas (Fongaro et al., 2021). Em termos de vírus viável, o tempo necessário para a inativação de 90% (T_{90}) do SARS-CoV-2 em temperatura ambiente (20-26 ºC) em águas residuais variou de 1,2 a 1,6 dias. Já com relação ao T_{90} do RNA viral nas mesmas condições, esse tempo foi de 3,3 a 12,6 dias, ou seja, o RNA viral persiste no ambiente por mais tempo que o vírus, o que indica que a quantificação do RNA viral no efluente não necessariamente implica na presença do vírus viável ou com o risco de infecção, tal como sugerido por Wurtzer et al. (2021), Mahlknecht (2022) e Atoui et al. (2023). Apesar desses fatores, conforme Bivins et al. (2020), o vírus permaneceu infeccioso por 7 dias em águas residuais (20 °C, RT).

Ainda segundo os estudos avaliados e sintetizados na tabela 1, outro fator importante para a persistência viral é o teor de impurezas da água, já que a inativação do SARS-CoV-2 viável, em uma mesma faixa de temperatura (20 - 26ºC), é mais rápida na água mineral, na água do mar filtrada, na água residual, e na água fluvial do que na água potável, ou seja, ocorre mais rapidamente em matrizes complexas do que em matrizes mais simples. Isso evidencia a necessidade de se considerar a composição físico-química e biológica da água, bem como o efeito de detergentes, enzimas e filtragem de partículas na realização de ensaios de persistência (Mahlknecht, 2022). Isto, pois, fatores como sólidos dissolvidos e pH podem alterar a persistência do SARS-CoV-2, sendo que uma menor razão entre sólidos voláteis/sólidos totais (ex. água fluvial) aumenta a adsorção viral dos sólidos e, consequentemente, aumenta a persistência viral (Picó & Barceló, 2023). Além disso, sabe-se que a filtragem e o tratamento adequado das águas contaminadas com o vírus, RNA viral ou partículas virais reduz a concentração desses poluentes nas diversas matrizes de água, evitando a difusão do patógeno e a sua interação com os contaminantes ambientais pré-existentes (Mahlknecht, 2022). Ainda, Rimoldi et al. (2020), ao fazerem a avaliação preliminar da infectividade do vírus SARS-CoV-2 em águas residuais e rios de Milão, encontraram que ela era nula em ambos. Devido a essas características, Wurtzer et al. (2021) consideram que a avaliação de risco para infecção viral por meio de águas residuais poderia ser mais realista usando o isolamento sistemático da cultura de células ou um ensaio baseado em integridade.

Tabela 1. Estimativa do parâmetro T_{90} para cada uma das amostras em diferentes matrizes de água (água potável, água residual, água fluvial, água mineral e água do mar), brutas ou filtradas, e em diferentes temperaturas, conforme o estudo mencionado.

Referência	Vírus	Matriz	Temperatura	T_{90}
Ahmed et al. (2020)	RNA viral	Água potável	4 ºC	58,6 dias
			25 ºC	15,2 dias
		Água residual bruta	4 ºC	27,8 dias
			25 ºC	12,6 dias
		Água residual autoclavada	4 ºC	43,2 dias
			25 ºC	13,5 dias
Bivins et al. (2020)	SARS-CoV-2	Água potável	20 ºC	2,0 dias
		Água residual bruta AT	20 ºC	1,6 dias
		Água residual bruta BT	20 ºC	2,1 dias
	RNA viral	Água potável	20 ºC	33,0 dias
		Água residual bruta AT	20 ºC	3,3 dias
		Água residual bruta BT	20 ºC	26,0 dias
Oliveira et al. (2021)	SARS-CoV-2	Água fluvial	4 ºC	7,7 dias
			24 ºC	1,9 dias
		Água fluvial filtrada	24 ºC	3,3 dias
		Água residual bruta	4 ºC	5,5 dias
			24 ºC	1,2 dias
		Água residual filtrada	24 ºC	1,5 dias
Sala-Comorera et al. (2021)	SARS-CoV-2	Água fluvial filtrada	4 ºC	3,8 dias
			20 ºC	2,3 dias
		Água do mar filtrada	4 ºC	2,2 dias
			20 ºC	1,1 dias
	RNA viral	Água do mar filtrada	20 ºC	16,6 dias
		Água do mar não-filtrada	20 ºC	8,9 dias
Yang et al. (2022)	RNA viral	Água residual bruta	4 ºC	17,2 dias
		Água residual bruta	26 ºC	7,68 dias
Pitol et al. (2023)	SARS-CoV-2	Água mineral	25 ºC	43,45 horas
Sherchan et al. (2023)	SARS-CoV-2	Água residual bruta	37 ºC	10,4 horas
		Água residual filtrada	37 ºC	10,8 horas
		Água residual secundária	37 ºC	18,3 horas

Legenda: Água residual: água coletada diretamente do sistema de coleta de esgoto. Água residual filtrada: água originária do esgoto e que passou pela filtração como um primeiro tratamento. Água residual secundária: água resultante do processo completo de tratamento de esgoto, antes de ser despejado nos rios. AT: os pesquisadores utilizaram, comparativamente, uma alta concentração de vírus na solução utilizada para inoculação (10^5 $TCID_{50}$/mL). BT: os pesquisadores utilizaram, comparativamente, uma baixa concentração de vírus na solução utilizada para inoculação (10^3 $TCID_{50}$/mL).

Ao se considerar os estudos de persistência viral e que em vários países menos de 30% do esgoto gerado é tratado antes de ser descarregado nos córregos (Rodriguez et al., 2020), que o vírus não é totalmente eliminado em ETEs convencionais (Picó & Barceló, 2023), que mais de 70% da superfície terrestre é coberta por água (WSS, 2019) e a magnitude da pandemia de COVID-19 (Gonzaga et al., 2020; Barlow et al., 2021), há uma preocupação quanto aos possíveis impactos aos seres vivos, principalmente de organismos aquáticos, em decorrência da exposição ao vírus, quanto pela presença de partículas virais na água. Ainda, considerando que o RNA do SARS-CoV-2 tende a permanecer no lodo das ETEs ou nas águas residuais não tratadas (Pourakbar et al., 2022) e pode permanecer por até 33,0 dias no ambiente, a interação desse material genético com a microbiota do lodo também pode induzir o surgimento de microrganismos e variantes mais resistentes aos sanitizantes. No entanto, ainda não há pesquisas que avaliem o efeito da interação desses poluentes com o SARS-CoV-2 e/ou partículas virais sobre a biota.

Estudos sobre a avaliação da toxicidade dos peptídeos SARS-CoV-2 em organismos aquáticos ajudaram a esclarecer sobre o potencial ecotoxicológico de fragmentos peptídicos do vírus em organismos não humanos. Constatou-se nesses estudos que a interação entre estruturas do SARS-CoV-2 e organismos aquáticos pode, por si só, induzir respostas biológicas negativas variadas, marcadas pelo aumento de biomarcadores preditivos de estresse oxidativo e a alteração colinesterásica [girinos de *Physalaemus cuvieri* (Fitzinger, 1826), família Leptodactylidae] (Charlie-Silva et al., 2021; Silva et al., 2021)] e alterações locomotoras e no comportamento olfativo [*Culex quinquefascitus* (Say, 1823), família Culicidae] (Mendonça-Gomes et al., 2021). Além disso, alterações no crescimento/desenvolvimento, instabilidade genômica e danos ao DNA foram reportadas em *Poecilia reticulata* (Peters, 1859), família Poeciliidae (Malafaia et al., 2022; Gonçalves et al., 2022), bem como diversas alterações morfológicas e na resposta imune em *zebrafish* [*Danio rerio* (F. Hamilton, 1822), família Cyprinidae] (Fernandes et al., 2022; Tyrkalska et al., 2023).

Por outro lado, menor atenção tem sido dedicada aos potenciais efeitos da contaminação aquática pelo SARS-CoV-2 em organismos terrestres. Estes organismos, em geral, foram utilizados para avaliar a sua suscetibilidade à infecção viral e seus papéis na disseminação do COVID-19 (Tiwari et al., 2020; Audino et al., 2021; Delahay et al., 2021). Ainda, Luz et al. (2022), em um estudo que visou avaliar os efeitos diretos causados a camundongos machos (Swiss e C57Bl/6J) pela exposição direta (intraperitoneal) de fragmentos peptídicos do SARS-CoV-2 evidenciou que houve efeitos cerebrais nos camundongos levando à déficit de memória e que a linhagem C57Bl/6J é mais sensível a essa exposição. Apesar desse estudo ter demonstrado que há efeitos ecotoxicológicos também em mamíferos terrestres, essa exposição não ocorre naturalmente. Ainda, para inferir impactos ecológicos com maior certeza, dever-se-ia realizar uma análise geracional e de *fitness*, incluindo também a avaliação de filhotes. Assim, os relatos de Yang et al. (2022), Pitol et al. (2023), Javanbakht et al. (2024) e Tandukar et al. (2024) sobre a permanência do RNA viral em corpos d'água, além das evidências de Luz et al. (2022), justificam a realização dos estudos sobre os possíveis efeitos ecotoxicológicos nos organismos aquáticos e terrestres, mesmo após o fim da pandemia da COVID-19 e, consequentemente, a redução do risco de contaminação direta. Além disso, o SARS-CoV-2 representa um risco ecotoxicológico para a biota, podendo impactar a dinâmica e distribuição de suas populações (Luz et al., 2022), alertando também sobre a necessidade de uma avaliação ecológica sobre este tema.

2.4 ESTUDOS ECOTOXICOLÓGICOS COM CAMUNDONGOS

Em ecotoxicologia, os estudos laboratoriais são importantes para elucidar mecanismos de ação tóxica decorrentes da contaminação ambiental e que podem resultar em alterações comportamentais e fisiológicas de seres vivos. Como os sistemas naturais são complexos e dinâmicos, estudos experimentais permitem o controle de inúmeras variáveis existentes em sistemas naturais, auxiliando na verificação de um fator como causa de um determinado efeito, além de fornecer percepções da sua relação com processos individuais, populacionais, comunitários, ecossistêmicos e evolutivos (Ford et al., 2021). Ainda, podem prever comportamentos ecologicamente importantes no campo (Fisher et al., 2015), inclusive a aptidão de indivíduos na natureza (Moiron et al., 2020).

Testes ecotoxicológicos *in vivo* são fundamentais para compreender os impactos em organismos vivos por substâncias potencialmente perigosas em seus habitats naturais (Luan et al., 2020; Pastorino et al., 2022), permitindo a avaliação da extensão de um impacto ecológico. Em geral, modelos experimentais (roedores, coelhos e outros animais vertebrados) são utilizados principalmente por serem opções econômicas, de ciclo de vida curto, alta taxa de reprodução, mais simples de manusear, repetíveis e com menos conflitos éticos em comparação com organismos selvagens (Rosner et al., 2023; Ferreira et al., 2024). Ainda, os testes em animais constituem um método confiável para prever consequências adversas em humanos e no ambiente (Zeidi et al., 2023).

Estudos ecotoxicológicos com camundongos são bastante utilizados para elucidar mecanismos e consequências provenientes de contaminação ambiental, pois podem servir de modelo para pequenos mamíferos terrestres, além de fornecer *insights* sobre como um poluente pode interferir na saúde humana, dada a nossa semelhança genética (99%) (Garcia, 2006). Assim, podem fornecer subsídios para prever alterações comportamentais, fisiológicas e de aptidão, além de evidenciar possíveis processos ecológicos importantes que estejam ocorrendo desde a nível individual a ecossistêmico. O camundongo [*Mus musculus* (Linnaeus, 1758); família Muridae] é um mamífero pequeno, muito prolífero, tem período de gestação curto, é de fácil domesticação e manutenção (Chorilli et al., 2007). É uma espécie cosmopolita e de hábitos noturnos, se acomodando em uma grande variedade de condições ambientais, desde que seja de tamanho apropriado às suas necessidades (Santos, 2006), sendo, por isso, uma espécie considerada como uma praga urbana quando em meio natural.

Mullick et al. (2020) ressalvam que animais comuns de experimentação (ex. camundongos, ratos, coelhos, porcos-da-índia) não são naturalmente sensíveis ao SARS-CoV-2 por não possuírem receptores ACE2, o que limita a sua utilização em experimentos voltados para o estudo da COVID-19 e ao desenvolvimento de medidas profiláticas ou de tratamento. No entanto, como (a) o presente trabalho é voltado para a ecotoxicologia; (b) os camundongos são animais amplamente estudados; (c) objetivamos utilizar os camundongos como modelos para pequenos mamíferos terrestres, dada a sua maior viabilidade em comparação a animais selvagens; além de que (d) a simulação em laboratório com camundongos facilitaria a replicação do experimento em outras localidades, o uso de camundongos é recomendável.

Neste estudo, utilizamos fêmeas de camundongo da linhagem C57BL/6J como modelo experimental, já que os camundongos dessa linhagem são isogênicos, o que reduz a influência da diversidade genética no resultado observado. Além disso, como estudos anteriores já evidenciaram que camundongos da linhagem C57Bl/6J são mais sensíveis às proteínas virais do SARS-CoV-2 (Luz et al., 2022) e que a família Coronaviridae infecta principalmente mamíferos (Manzini et al., 2021), esse modelo experimental pode esclarecer efeitos desconhecidos causados à biodiversidade pela exposição ao SARS-CoV-2 por meio da ingestão de água contaminada durante a sua maturação sexual, principalmente aos pequenos mamíferos terrestres, inclusive com relação à possíveis impactos gerados à sua capacidade reprodutiva e comportamental. Assim, pode-se avaliar se essa exposição poderia impactar diretamente o *fitness* dessa espécie e a sua viabilidade populacional em meio natural.

As camundongos fêmeas foram obtidas a partir de matrizes provenientes do Biotério do Laboratório de Toxicologia Aplicada ao Meio Ambiente (LabTox) do Instituto Federal Goiano (IF Goiano), Campus Urutaí (GO, Brasil). No período *in vivo*, os espécimes foram mantidos em caixas, a 23ºC e 50% de umidade relativa do ar, em ciclo normal claro/escuro, com ração e água oferecidos *ad libitum*, além da limpeza, manutenção e monitoramento das caixas sendo realizados a cada 3 dias. Esta pesquisa foi desenvolvida em parceria estabelecida entre a Universidade Federal de Uberlândia e o Instituto Federal Goiano - Campus Urutaí, tendo aprovação pela Comissão de Ética no Uso de Animais (CEUA) do Instituto Federal Goiano sob o protocolo n. 21/2022-R1.

2.5 CONSIDERAÇÕES FINAIS

Apesar do conceito de saúde que a OMS tem tentado estabelecer desde 1978, as nações usualmente se restringem aos cuidados biomédicos, isolados e mecanicistas do cuidado à saúde, negligenciando dimensões importantes como o bem-estar ecológico. Nesse sentido, a perspectiva da Uma Só Saúde é uma promissora ferramenta auxiliar e integradora de saberes, fornecendo uma visão panorâmica sobre a realidade ambiental e suas interconexões com a saúde ambiental, animal e humana.

A partir do levantamento bibliográfico realizado, evidenciou-se que o SARS-CoV-2 influencia negativamente tanto humanos, quanto organismos aquáticos e terrestres. Embora relacionado primariamente a doenças respiratórias e entéricas, há evidências de que esse vírus ou suas partículas virais são capazes de alterar severamente a fisiologia e o comportamento dos organismos expostos, podendo, inclusive, influenciar a sua saúde reprodutiva. Deste modo, o vírus tem o potencial de influenciar o *fitness* dos indivíduos, impactar as populações e alterar o equilíbrio e dinâmicas ecossistêmicas futuramente. Apesar desses relatos, as pesquisas realizadas apenas estavam vinculadas à exposição de partículas virais da cepa original do SARS-CoV-2, o que limita a compreensão sobre os potenciais efeitos resultantes do vírus viável e de variantes emergentes e recombinantes. Ademais, as investigações focaram principalmente em organismos aquáticos, por habitarem o meio em que há uma prevalência da exposição proveniente de águas residuárias. Todavia, há a possibilidade de a água ser um meio de contaminação de animais terrestres, caso ainda não avaliado. Ainda, as exposições previamente realizadas em camundongos, um modelo para pequenos mamíferos não-humanos terrestres, não refletiam uma exposição que realmente ocorreria na natureza, pois utilizaram a via intraperitoneal.

Embora haja poucas evidências sobre a contaminação secundária de COVID-19 ocasionada em sinergia à contaminação da água, há a possibilidade, por exemplo, de que animais considerados pragas (roedores, baratas, pombos, moscas, pernilongos, etc), por viverem em ambientes altamente expostos a esse tipo de contaminação, também possam atuar como disseminadores ambientais do vírus e ser, portanto, possíveis pivôs de surtos. Esta possibilidade somada à transmissão zoonótica reversa a gatos e furões, bem como os efeitos relativos à propagação de partículas virais a animais, abre a expectativa para consequências ambientais ainda desconhecidas e potencialmente perigosas à saúde pública e em uma abordagem integrada para prevenção à propagação de epidemias. Assim, há a necessidade de pesquisas para elucidar a possibilidade de efeitos ecotoxicológicos a animais terrestres, visando elucidar as lacunas de conhecimento existentes.

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3 BEYOND THE VIRUS: ECOTOXICOLOGICAL AND REPRODUCTIVE IMPACTS OF SARS-CoV-2 LYSATE PROTEIN IN C57BL/6J FEMALE MICE

ABSTRACT

Since the establishment of the COVID-19 pandemic, a range of studies have been developed to understand the pathogenesis of SARS-CoV-2 infection, vaccine development, and therapeutic testing. However, the possible impacts that these viruses can have on non-target organisms have been explored little, and our knowledge of the consequences of the COVID-19 pandemic for biota is still very limited. Thus, the current study aimed to address this knowledge gap by evaluating the possible impacts of oral exposure of C57Bl/6J female mice to SARS-CoV-2 lysate protein (at 20 µg/L) for 30 days, using multiple methods, including behavioral assessments, biochemical analyses, and histopathological examinations. Although we did not have evidence of hematological, mutagenic, or genotoxic effects, we noted that the ingestion of SARS-CoV-2 lysate protein-induced behavioral disorders (hypoactivity, anxiety-like behavior, and short-term memory deficit), which were associated with oxidative stress and dopaminergic and cholinesterase imbalance in the animal brain. Furthermore, the elevation of bilirubin levels and lactate dehydrogenase levels in these animals suggests the occurrence of hepatic changes, and the redox imbalance, nitrosative stress, and elevated production of IFN-γ and inflammatory infiltration in the duodenum, disrupted follicular structure, and presence of vacuoles in granulosa cells, in ovarian, indicate that the SARS-CoV-2-exposed group showed significant toxicity. Principal component analysis (PCA) and cluster analysis confirmed that the groups were clearly separated and showed that the largest changes upon SARS-CoV-2 exposure were related to ROS, MDA, nitrite, IFN-γ/IL-10 levels and SOD and catalase activity in the ovary; IFN-γ/IL-10 production and SOD activity in the duodenum; BChE activity in the brain; bilirubin levels and lactate dehydrogenase activity in the serum; number of primary follicles in the ovary. In conclusion, our study provides new insights into the toxicity of SARS-CoV-2 lysate proteins in a non-target terrestrial organism of infection and, therefore, expands our understanding of the real extent of the ecological/environmental impact of the COVID-19 pandemic.

Keywords: Histopathology, SARS-CoV-2 lysate protein, oxidative stress, non-target organisms, ecotoxicology, biomarkers.

3.1 INTRODUCTION

COVID-19 is a highly transmissible infectious disease whose etiological agent is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; Coronaviridae family) (Cucinotta & Vanelli, 2020; WHO, 2021). The SARS-CoV-2 virus was first reported in a pneumonia epidemic in Wuhan, Hubei, China, on December 31, 2019 (Huang et al., 2020), having managed to spread and evolve into a pandemic within a few months, causing several countries' health systems collapse (Gonzaga et al., 2020; Barlow et al., 2021). Therefore, with its spread, COVID-19 had biomedical, epidemiological, social, economic, political, cultural, and historical repercussions (Gomes et al., 2020; Naseer et al., 2023; Khan et al., 2024). Initially, global social isolation measures were used, such as quarantine and lockdown (Lima et al., 2020); but, with the development of research on various aspects of the disease, it was possible, throughout the pandemic, to use more effective prophylactic methods, such as vaccines (Fragkou & Dimopoulou, 2021; Ghasemiyeh et al., 2021; Krammer, 2023).

Among humans, the classic form of transmission of SARS-CoV-2 occurs through the air and/or through contact with infected people (Harrison et al., 2020; Meyerowitz et al., 2020). It is also known that the virus can persist in the environment on surfaces made of metallic, vitreous, and plastic materials for up to 9 days (Kampf et al., 2020; Ashokkumar et al., 2023). Furthermore, several studies have already reported the presence of SARS-CoV-2 in feces, urine and anal swabs from infected patients (Lin et al., 2021; Timpe et al., 2021; Liu et al., 2021; Termansen & Frische, 2023; Gandini et al., 2024), with its genetic material identified in domestic and hospital sewage from different locations (Kumar et al., 2020; Weidhaas et al., 2021; Crank et al., 2022; Mogili et al., 2024). Therefore, as highlighted by Guerrero-Latorre et al. (2020), Rimoldi et al. (2020) and Mahlknecht et al. (2021), there was a significant input of the virus into river ecosystems, mainly in countries with a lack or scarcity of basic sanitation (Guerrero-Latorre et al., 2020; Pandey et al., 2021; Taniwaki et al., 2024).

In this sense, the potential ecotoxicological effect of SARS-CoV-2 on non-target organisms has emerged as a relevant issue, especially since the virus can cause the death of exposed organisms or negatively impact their growth, development, and/or reproduction (Charlie-Silva & Malafaia, 2021). Studies that sought to evaluate this issue showed that the interaction between the structures of SARS-CoV-2 and non-target aquatic organisms can, in itself, induce negative biological responses, although the development of an infectious condition such as in humans has not been observed (Charlie-Silva et al., 2021; Mendonça-Gomes et al., 2021; Gonçalves et al., 2021; Fernandes et al., 2022; Freitas et al., 2022; Gonçalves et al., 2022; Kraus et al., 2022; Luz et al., 2022; Malafaia et al., 2022; Freitas et al., 2023). The species evaluated in these studies presented varied effects, mainly marked by the induction of oxidative stress and cholinesterase alteration [tadpoles of *Physalaemus cuvieri* (Charlie-Silva et al., 2021; Silva et al., 2021)] and olfactory locomotor alterations in *Culex quinquefascitus* larvae (Mendonça-Gomes et al., 2021). Furthermore, changes in growth/development, genomic instability, and DNA damage have been reported in *Poecilia reticulata* (Malafaia et al., 2022; Gonçalves et al., 2022), as well as morphological changes and in the immune response of adult zebrafish (*Danio rerio*) (Fernandes et al., 2022; Tyrkalska et al., 2023).

In non-human mammals, most studies have focused on the susceptibility of different species to viral infection and their possible uses as experimental models for the study of new therapeutic approaches to COVID-19 (Shi et al., 2020; Tiwari et al., 2020; Audino et al., 2021; Biancatelli et al., 2021; Delahay et al., 2021; Melo et al., 2022; Li et al., 2024). Intranasal inoculation of SARS-CoV-2 into WT-HB-01 and hACE2 male mice, for example, resulted in the infection only of mice that had human receptors (Abdel-Moneim & Abdelwhab, 2020), which was accompanied by the induction of weight loss, lung lesions and histopathological changes (interstitial pneumonia) in animals. On the other hand, Luz et al. (2022) showed that, after 24 h of intraperitoneal administration of peptide fragments from SARS-CoV-2, Swiss and C57Bl/6J male mice showed memory deficits, suppression of the activity of acetylcholinesterase (AChE), superoxide dismutase (SOD) and catalase (CAT) in the brain, showing greater sensitivity of C57Bl/6J mice. In Oh et al. (2022), the authors reported that injection of SARS-CoV-2 spike protein S1 subunit (S1 protein) into mouse hippocampus induced cognitive deficit and anxiety-like behavior. Fontes-Dantas et al. (2023) observed that the brain infusion of recombinant Spike protein impacted the cognition of male-induced Swiss mice, and stress-related behavior was observed in female C57Bl/6J mice tail vein injected with the different Spike peptides (S1 full-length subunit and truncated S1 subunit) (Nuovo et al., 2021). Furthermore, Zeng et al. (2022) and Li et al. (2024) observed that C57Bl/6J and hACE2-All CDS-B6J mice presented distinct histopathological changes in the gastrointestinal system (mainly in the duodenum, stomach, and jejunoileum) upon abdominal stimulation of SARS-CoV-2 Spike protein. Therefore, such studies reinforce the ecotoxicological potential of SARS-CoV-2 on non-target organisms and show that its effects are not restricted to aquatic animals.

However, it is important to highlight that the studies mentioned above did not evaluate whether the ingestion of water contaminated with SARS-CoV-2 (with all its structures) – i.e., a more plausible route in ecological terms – represents a risk to the health of terrestrial organisms' non-targets. In response to these concerns, the current study aimed to address this knowledge gap by evaluating the possible impacts of oral exposure of female C57Bl/6J mice to SARS-CoV-2 lysate protein, simulating their ingestion via contaminated water. We start from the hypothesis that the ingestion of SARS-CoV-2 lysate protein impacts the health of non-human terrestrial mammals (and non-targets of infection), causing behavioral, hematological, serological, mutagenic, genotoxic, biochemical, as well as histopathological changes. We believe that studies of this nature are crucial for advancing our understanding of the broader ecological and environmental impacts associated with the COVID-19 pandemic. Focusing on the oral exposure of non-target terrestrial organisms to SARS-CoV-2 lysate protein, this research provides new insights into how viral pollutants can affect ecosystems beyond their primary human health implications. Furthermore, our findings can inform the development of more effective mitigation and remediation strategies for managing the contamination of aquatic systems by SARS-CoV-2 and other viruses from the Coronaviridae family, which may pose long-term risks to aquatic and terrestrial species. This study thus contributes to a growing body of research on emerging viral pollutants and highlights the importance of ecological monitoring in the aftermath of global pandemics.

3.2 MATERIAL AND METHODS

3.2.1 SARS-CoV-2

In this study, we used SARS-CoV-2 lysate protein (SARS.CoV2/SP02.2020.HIAE.Br, original strain lineage B, GenBank: MT126808.1), kindly provided by Dr. Edson Luiz Durigon (Department of Microbiology, University of São Paulo, Brazil), having been isolated from the first confirmed case in Brazil. As described in Araújo et al. (2020), the virus was recovered from nasopharyngeal swab samples from patients diagnosed with COVID-19 (and symptomatic) who returned to São Paulo (Feb 2020) after traveling to the Lombardy region in Northwest Italy. After isolation, SARS-CoV-2 was propagated in Vero cells (E6, CCL-81, and hSLAM) and characterized by genomic sequencing, electron microscopy, and in vitro replicability [see details presented in Araújo et al. (2020)]. The viral stock solution was composed of supernatant from Vero E6 cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum diluted (1:5) in NucliSENS® lysis buffer. The determination of SARS-CoV-2 lysate protein concentration in stock solution (i.e., 0.9320 g viral protein/dL) was carried out using a commercial kit (kit BT1000900, lot 09S01A, Biotécnica Ind. e Com. Ltd., Varginha, Brazil). Furthermore, confirmation of the positivity of the stock solution for SARS-CoV-2 was carried out via chromatographic immunoassay using a commercial kit (Wondfo Biotech. Co. Ltd., USA).

3.2.2 ANIMALS AND EXPERIMENTAL DESIGN

As a model system representative of the group of terrestrial mammals that are not targets of SARS-CoV-2 infection, we used C57Bl/6J female mice (*Mus musculus*; family Muridae) obtained and maintained in the vivarium of the Laboratory of Toxicology Applied to Environment (Goiano Federal Institute - Urutaí Campus, Goiás, Brazil). The animals were kept in cages housed on ventilated shelves with controlled light (12h light/12h dark), temperature (24ºC ± 2ºC), and relative humidity (± 50%) and were offered food (standard for rodents) and water *ad libitum*. C57Bl/6J was the strain chosen because it is inbred, which reduces the influence of genetic diversity on its biological responses and is considered sensitive to exposure to SARS-CoV-2 peptides (Luz et al., 2022). We chose to use females, as they naturally exhibit less aggressive behavior than males (Van-Loo et al., 2003; Jirkof et al., 2020), avoiding any interpretative bias in their responses to the tests carried out.

Thus, 40 female mice (3.5-4 months) were distributed into two experimental groups (n=20 mice/group, n=10 animals/replicate). Each female was chosen randomly, weighed, and separated so the replicates would have the same age and initial biomass average. The "control" group was composed of animals unexposed to SARS-CoV-2 lysate protein and, in the "SARS-CoV-2" group, the animals were exposed to the novel coronavirus, for 30 days, orally, whose SARS-CoV-2 lysate protein was dispersed in drinking water offered to the animals via drinking fountain (to 20 µg/L). The animal cages were cleaned every three days, and the water in the drinking fountains was renewed. Such concentration was chosen based on the study by Ferreira et al. (2023) and Luz et al. (2023), being considered a predictive and environmentally relevant that simulates a pessimistic scenario of high discharge of domestic and hospital sewage, especially in regions with a high rate of infection. To date, the studies mainly express the concentrations of SARS-CoV-2 in aquatic environments from equivalent viral RNA (genome copies/mL). Throughout the experiment, weekly feed and water intake were recorded according to Malafaia et al. (2015).

3.2.3 TOXICITY BIOMARKERS

3.2.3.1 Behavioral Toxicity

To evaluate possible behavioral changes induced by SARS-CoV-2 lysate protein exposure, on the 29th experimental day, females were subjected to behavioral paradigms, following the order of execution from least stressful to most stressful (open field test → novel object recognition test → tail-suspension tests). Such tests were conducted in a specific room (with sound insulation, temperature, and lighting controlled), and between each session, the apparatus was cleaned with ethanol (at 70%). It is worth noting that, considering that the hormonal peaks of the females' estrous cycle influence their behavior (Mora et al., 1996; Lovick & Zangrossi Jr., 2021), the females' estrous phases (n=40; n=20/group) were determined at the end of the tests, according to Byers et al. (2012). Therefore, we considered only the females in the estrus and proestrus phases (totaling n=10 animals/group) because these phases were predominant in the individuals.

3.2.3.1.1 Open field test (OFT)

In the open field test (OFT), we evaluated possible locomotor changes and/or the induction of anxiety-like behavior associated with the consumption of water containing SARS-CoV-2 lysate protein, according to Guimarães et al. (2021). As discussed by Seibenhener and Wooten (2015), this test is based on the conflict between the rodents' innate behavior of exploring a new environment and the natural tendency to avoid a potentially dangerous area (open field). Briefly, the test consisted of introducing the animals individually into the center of a rectangular polypropylene arena with opaque white walls (dimensions: 16.5 cm high x 31 cm wide x 40.5 cm long) and filming them for 5 min. The locomotor activity of the animals was inferred by recording the number of quadrants crossed, plotted virtually on the computer screen, and the ratio between the percentage of time spent in the peripheral zone of the apparatus and the total number of quadrants covered was considered an indicator of anxiety-like behavior, like Estrela et al. (2021a).

3.2.3.1.2 Novel object recognition (NOR) test

The NOR test was performed to evaluate possible changes in the animals' short-term memory (Ennaceur & Delacour, 1988), based on the procedures described in Luz et al. (2022), with minor modifications. Briefly, the NOR test was performed in two sequential steps. In the first (training section), two identical objects [called familiar objects (FO right and FO left); LEGO® toys, blue in color and cubic in shape)] were introduced on opposite sides of the arena (the same one used in the OFT), 2 cm from the walls, allowing the animals to explore them freely for 5 min. Subsequently, in the test section, one of the objects was replaced by a novel object (NO) [of different size and shape], and the animals explored them for another 5 min. Notably, between the NOR test sessions, the NO and FO were placed in alternating positions, thus excluding potential influences of spatial preferences on the animals' response. The exploration time of objects was recorded only when the animals smelled them from a distance ≤ 2 cm or touched them intentionally (Rajagopal et al., 2014). At the end of the test, different indices were determined using the following equations (Ennaceur & Delacour, 1988; Hammond et al., 2004; Aubele et al., 2008; Oliveira et al., 2010; Gaskin et al., 2010; Antunes & Biala, 2012) which, when combined, provide estimations of discrimination, preference, retention, and/or recognition process.

Index 1 = $\mathrm{TNO} - \mathrm{TFO}$ Eq. 1

$$\text{Index } 2 = \frac{\text{TN} \quad - \text{TFO}}{\text{TNO} + \text{TFO}} \qquad \text{Eq. 2}$$

$$\text{Index } 3 = \frac{\text{TNO}}{\text{TNO} + \text{TFO}} \times 100 \qquad \text{Eq. 3}$$

where “TNO” is the exploration time devoted to the novel object and “TFO” is the time devoted to the familiar object. Index 2 results can vary between +1 and −1. A positive score indicates more time spent with the NO, a negative score indicates more time spent with the FO, and a zero score indicates a null preference (Antunes & Biala, 2012). Furthermore, index 3 above 50% indicates NO preference, below 50% FO preference, and 50% no preference (Hammond et al., 2004). Similarly, these indices were applied in the training session of the “control” group to validate the NOR test, replacing “TNO” with “TFOR” and “TFO” with “TFOL” in the equations above. “TFOR” represents the exploration time devoted to the FO right, and “TFOL” represents the time devoted to the FO left.

3.2.3.1.3 Tail-suspension test (TST)

The tail-suspension test (TST) was performed to evaluate the possible induction of depressive-like behavior by ingestion of SARS-CoV-2 lysate protein. As highlighted by Cryan et al. (2005), the TST is based on the observation that mice, after initial escape-oriented movements, develop an immobile posture when placed in a situation of hemodynamic stress by being hung uncontrollably by the tail. This immobility, according to Weingartner & Silberman (1982), is analogous to the clinical observations that depressed patients are unable or reluctant to maintain effort, reflected in a pronounced psychomotor impairment. In our study, we used an apparatus described by Can et al. (2012). Briefly, the test consisted of placing a plastic piece around the mouse's tail (reducing its ability to suspend its own body), attaching the animal's tail to the metal rod of the apparatus (with adhesive tape), and, subsequently, filming it for 5 min to record the time of immobility, like Lima et al. (2019).

3.2.3.2 Mutagenicity And Genotoxicity Biomarkers

3.2.3.2.1 Micronucleus test

To evaluate the possible mutagenicity associated with ingesting SARS-CoV-2 lysate protein, we performed the micronucleus test in peripheral blood, using the method of Hayashi et al. (1990), with some modifications. This test was chosen because it is widely used to evaluate the possible mutagenicity of different pollutants (Sommer et al., 2020; Rodrigues-

Tanamachi et al., 2021; Hernandez-Toledano et al., 2023). Briefly, 15 µL of peripheral blood (collected via a small incision at the tip of the tail) was mixed with 15 µL of phosphate-buffered saline (PBS) (pH 7.2). Of this solution, 5 µL was deposited on a coverslip, which was subsequently covered by a glass slide previously coated with 0.1% acridine orange solution (2 slides/sample). The slides were sealed with Canada balsam and left to rest under refrigeration (4ºC for 24h) to maximize staining. Then, the slides were analyzed under a fluorescence microscope equipped with a blue excitation filter and a green barrier filter (Figure 2). From the images obtained, we recorded the frequency of micronucleated polychromatic erythrocytes/2000 erythrocytes, according to Krishna and Hayashi (2000).

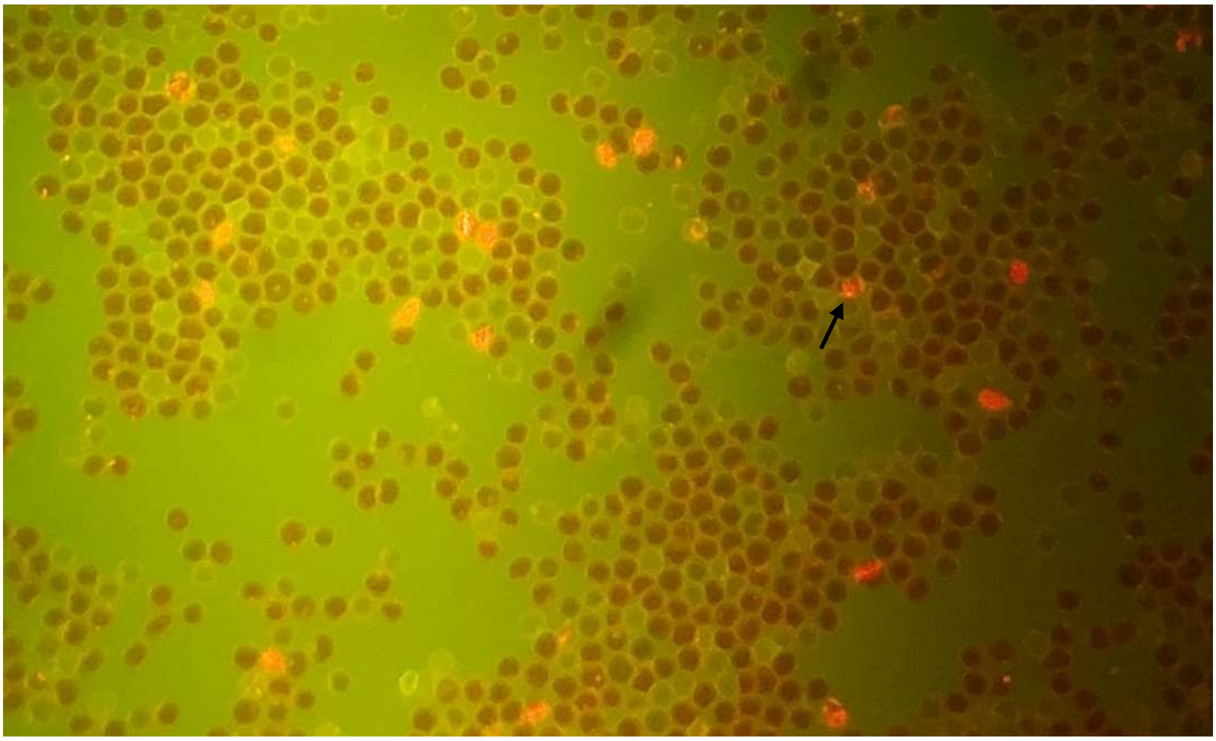

Figure 2. Photo of a micronucleus observed under a fluorescence microscope. In this analysis, we counted and classified 2000 erythrocytes per animal, which could be classified as normochromatic erythrocytes (brownish cells) or polychromatic erythrocytes (cells stained bright orange). We also assessed whether or not there was a micronucleus, a small greenish-yellow fluorescent structure inside them, as indicated by the arrow.

3.2.3.2.2 Comet assay

To evaluate the genotoxic potential of SARS-CoV-2 lysate protein, we performed the comet assay (or single-cell gel electrophoresis), which allows the evaluation of DNA damage related to single-strand breaks, lesions of labile alkaline sites, and/or incomplete repair sites, as well as crosslinks (Azqueta & Collins, 2013). In our study, the alkaline comet assay was performed with some modifications, per Singh et al. (1988). In summary, 5 µL of blood collected as already described (see item 2.3.2.1) were initially mixed with 50 µL of PBS. Then, 5 µL of this solution was mixed with 120 µL of low melting point agarose (0.5%) previously heated (37ºC), deposited on slides pre-coated with 1.5% standard agarose, and covered with a coverslip. (two slides were made per animal). After the agarose solidified at 4°C, the coverslip was carefully removed, and the slides were immersed in the dark in a lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris (hydroxymethyl) aminomethane at pH 10, with 1% Triton X-100 and 10% DMSO] for 2 h at 4ºC. Then, the slides were immersed in an alkaline buffer (0.3 mM NaOH and 1 mM EDTA; pH > 13; 30 min) to allow DNA binding and expression of the alkaline labile site. Electrophoresis was performed at 300 mA, 25V (0.90V/cm), for 30 min without light, using an electrophoresis vat cooled with ice (Poletta et al., 2008). After that, the slides were removed and neutralized with buffer solution (0.4M Tris-HCl, pH 7.5) for 5 min. After washing with purified water via reverse osmosis, the slides were fixed with absolute ethanol (10 min) and dried at room temperature.

The slides were stained with 30 µL of ethidium bromide solution (10 µg/L) and covered with a coverslip for immediate analysis under a fluorescence microscope (Figure 3). In total, 50 nucleoids/slide/animal were analyzed using CaspLab® software (v. 2.0), based on Gyori et al. (2014) and Liu & Liu (2024). The following parameters were used to evaluate the induction of possible DNA damage: (i) tail length (TL), (ii) percentage of DNA in the tail (Tail DNA%) and (iii) Olive tail moment (OTM), as described by Collins (2004) and adopted in previous studies (Ramos et al., 2016; Gonçalves et al., 2017; Fernandes et al., 2018; Estrela et al., 2021a; 2021b).

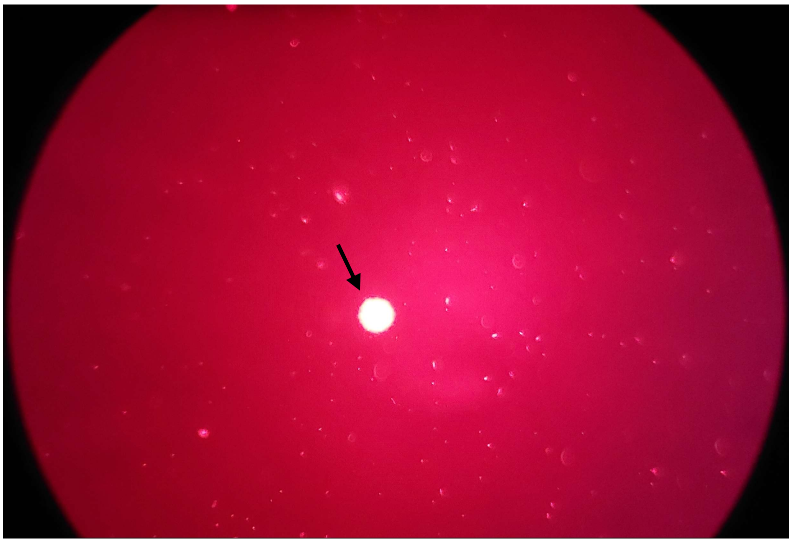

Figure 3. Photo of a nucleoid (indicated by the arrow) observed under the fluorescence microscope and evaluated in CaspLab® to the Comet assay analysis. In mammals, as red blood cells do not have a nucleus, the nucleoid obtained from this assay retains its round shape.

3.2.3.3 Predictive Biomarkers Of Hematological And Biochemical Changes

3.2.3.3.1 Hematological and serological analysis

The possible induction of hematological changes was assessed using the hemogram test, commonly used to assess the general health of individuals, including mice (Stiene-Martin et al., 1998; Restell et al., 2014). This test assesses the production of all blood cells, the oxygen transport capacity of the blood, the immune system, and other blood dyscrasias (George-Gay & Parker, 2003). In our study, the hemogram test was performed on five randomly selected individuals from each experimental group. For this, 100 µL of blood was collected (via decapitation) and mixed with K_2-EDTA in conical bottom microtubes, which were stored under refrigeration until analysis via a veterinary hematological analyzer (model Prokan PE-6800 VET, Prokan Electronics, Shenzhen, China). Furthermore, blood smears were prepared and stained with a fast Panoptic Kit (Laborclin, Pinhais, Paraná, Brazil) [like Kozima et al. (2020)] for differential leukocyte counting under an optical microscope, according to Russel et al. (1951). The additional blood was stored in microtubes and centrifuged at 672 ×*g* for 10 min at 4°C. The serum was separated and frozen at -80°C to determine the biomarkers summarized in Table 2.

3.2.3.3.2 Tissue biochemical biomarkers

3.2.3.3.2.1 Sample preparation

After euthanasia of the animals, fragments of different tissues/organs (brain, duodenum, liver, mesenteric lymph node, ovary, lungs, and kidneys) were collected and transferred to maceration tubes containing 500 µL of PBS (pH 7.2; 4°C) and five stainless steel beads (diameter: 2.8 mm). Then, the tubes were introduced into a cell and tissue disruptor (L-Beader® v.6) and processed according to the manufacturer's specific instructions for each organ/tissue. After complete maceration, 500 µL of PBS was added, homogenization was carried out in a vortex shaker, and subsequently, the samples were centrifuged at 1,136 × *g* for 10 min, at 4ºC. Then, the supernatants were collected and filtered with sterile syringe filters (pore: 0.45 µm) and stored at -80ºC until use.

Table 2. Summary of biomarkers evaluated in the serum of C57Bl/6L female mice unexposed or exposed to SARS-CoV-2 lysate protein.

Biomarkers	**Brief justification for choosing the biomarker**	**Methods**	**References**	**Reference code of the kits**
Total protein levels	These were assessed as they provide a baseline for normalizing other biochemical markers.	Biuret method	Gornall et al. (1949)	#BT1000900
Total cholesterol levels	Cholesterol levels are commonly used to assess lipid metabolism and potential hepatotoxicity, as alterations can indicate liver dysfunction.	Liebermann–Burchard Colorimetric Method	Abell et al. (1952)	#BT1000400
Albumin levels	Albumin is an essential protein synthesized in the liver, and its levels can indicate both nutritional status and liver function.	Bromocresol green method	Lehane et al. (1978)	#BT1000200
Triglyceride levels	Triglycerides are another key lipid biomarker that indicates the state of lipid metabolism. Variations in their levels may be associated with disruptions in metabolic processes.	Enzymatic method	Sullivan et al. (1985)	#BT1001000
Creatinine levels	Creatinine is a waste product from muscle metabolism and a standard renal function marker. Evaluating creatinine ensures that the exposure does not induce renal damage, which is important for overall health assessment.	Jaffe method	Bonsnss & Taussky (1945)	#BT1000700
Bilirubin levels	Elevated bilirubin levels can be a sign of liver dysfunction, specifically regarding the metabolism and clearance of heme. It is a crucial marker for	Photoelectric colorimetric method	Malloy & Evelyn (1937)	#BT1000300

	detecting hepatocellular damage, which could be induced by SARS-CoV-2 lysate.			
Alpha-amylase activity	Alpha-amylase is typically a marker of pancreatic and salivary gland function. Changes in its activity can indicate systemic metabolic disturbances, which might result from exposure to viral proteins.	2-chloro-4-nitrophenyl-beta-D-maltoheptaoside method	Hafkenscheid & Hessels (1985)	#BV9001800
Gamma-glutamyl transferase activity	GGT is an enzyme involved in glutathione metabolism and is commonly used to evaluate liver health, particularly for early detection of hepatic injury or stress.	Schumann method	Schumann et al. (2002)	#BT1100600
Alkaline phosphatase activity	ALP is a marker of liver and bone health. Its activity provides insights into liver function and can indicate cholestasis or other hepatic conditions.	p-nitrophenyl phosphate method	Neumann & Van Vreedendaal (1967)	#BT1106400
Lactate dehydrogenase activity	LDH is a marker of tissue damage, particularly in the liver, muscles, and heart. Elevations in LDH activity suggest cellular injury and are critical for detecting the cytotoxic effects of SARS-CoV-2 lysate.	Reduced diphosphonucleotide method	Wróblewski & Ladue (1955)	#BT1100400

#All commercial kits were obtained from Biotécnica Ind. e Com. Ltd., Varginha, Brazil.

3.2.3.3.2.2. Oxidative, nitrosative stress, and antioxidant activity biomarkers

In this study, we evaluated in samples from all tissues/organs collected the reactive oxygen species (ROS) and nitrite levels, considered the main reactive species in cells (Wink et al., 1995; Bhattacharya, 2015), according to procedures described in Zhao et al. (2013) and Bryan (2007), respectively. To evaluate the possible effect of ingesting water containing SARS-CoV-2 lysate protein on the enzymatic antioxidant system, the activity of superoxide dismutase (SOD) and catalase (CAT) was determined, according to Sinha et al. (1972) and Del-Maestro & McDonald (2018), respectively. Furthermore, malondialdehyde (MDA) levels were measured, considered the main aldehyde resulting from lipid oxidation and, therefore, a marker of oxidative stress (Moazamian et al., 2015). The results of these evaluations were relativized with the level of total proteins (evaluated via commercial kit - #BT1000900, Biotécnica Ind. e Com. Ltd., Varginha, Brazil) in each sample analyzed.

3.2.3.3.2.3 Biomarkers of changes in the cholinesterasic and dopaminergic systems

Aiming to infer the potential cholinesterasic or anti-cholinesterasic effect resulting from the ingestion of water containing SARS-CoV-2 lysate protein, the brain activity of the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was evaluated, according to Ellman et al. (1961). As highlighted by Silman and Sussman (2008), such enzymes participate in the hydrolysis of acetylcholine (ACh) at cholinergic synapses. On the other hand, the dopamine levels in the brain were evaluated to infer the treatment's possible effect on the dopaminergic system. To do this, we used a sensitive colorimetric detection of dopamine based on the enhanced-oxidase mimicking activity of cerium (IV), according to Liang et al. (2021).

3.2.3.3.3 Pro- and anti-inflammatory response biomarkers

To evaluate the pro- and anti-inflammatory response of mice exposed to SARS-CoV-2 lysate protein, interferon-gamma (IFN-γ) and interleukin-10 (IL-10) levels were determined. While IFN-γ is responsible for mediating inflammatory immune responses (Boehm et al., 1997), IL-10 acts to suppress/attenuate inflammatory processes (Zou & Secombes, 2016; Sakai et al., 2021). The target organs of these evaluations were defined based on ROS and nitrite production results to relate oxidative and nitrosative stress to possible inflammatory processes. Therefore, the production of IFN-γ and IL-10 was evaluated in the ovary and duodenum of the animals. Enzyme-linked immunosorbent assay (ELISA) kits purchased from PeproTech (São Paulo, Brazil) were used, following the manufacturer's instructions.

3.2.3.3.4 Histopathological analysis

To explore the potential histopathological effects induced by the ingestion of SARS-CoV-2 lysate protein, the entire right ovary and a segment of the duodenum (n=10 mice/group) were collected immediately after euthanasia and fixed in 10% neutral-buffered formalin for at least 72 h and posteriorly processed according to Wang et al. (2014), Zhanmu et al. (2020), and Al-Mahmood et al. (2021), with some modifications. Briefly, after fixation, the samples were washed in running water for 30 min to remove any residual formalin, then dehydrated through a graded series of ethanol concentrations (70%, 80%, 90%, and two changes of 100%), with each step lasting approximately one hour. The tissues were cleared in xylene (three changes of 30 min each) until transparency was achieved, followed by infiltration with paraffin wax at 58-60°C for three one-hour cycles. Once infiltrated, the tissues were embedded in paraffin blocks using standard embedding molds and allowed to solidify at room temperature. Sections of 5 µm were cut using a semi-automatic microtome, floated on a water bath at 45°C to remove folds, and mounted on glass slides. The sections were then stained with hematoxylin and eosin (H&E) using standard staining protocols for a detailed examination of tissue morphology, like Al-Mahmood et al. (2021).

First, the tissue sections were deparaffinized by immersion in xylene for two 5-minute intervals, followed by rehydration through a series of ethanol concentrations (100%, 90%, 80%, and 70%), each for approximately 3 min. Depending on tissue density, the sections were immersed in hematoxylin for 5-10 minutes and rinsed in tap water until the excess stain was removed and the nuclei were visible. Following the hematoxylin staining, the slides were counterstained in eosin for 30 s to 1 min, providing a contrasting stain for the cytoplasm. After staining, the sections were dehydrated through graded alcohols (70%, 80%, 90%, and 100%), cleared in xylene, and then mounted with a coverslip using a permanent mounting medium. All histological slides were analyzed using an Aperio ScanScope® CS microscope slide scanner and Aperio ImageScope software (version 12.4.6.5003, Leica), like Deligiannis et al. (2024) and Luissint et al. (2024). According to Pedersen & Peters (1968) and Hilal et al. (2020), we performed follicle quantification in the ovary. Like Sandrock et al. (2009), only ovaries in estrus (phase before ovulation) were selected for serial sections along the longitudinal plane. Primordial follicles were considered those oocytes surrounded by one layer of flattened granulosa cells, and primary follicles, oocytes surrounded by one layer of cuboidal granulosa cells. On the other hand, secondary follicles were counted when two or three layers of cuboidal granulosa cells surrounded the oocytes without antral space. Antral follicles contained more than four layers of granulosa cells with one or more independent antral spaces or a cumulus granulosa cell layer. The number of corpus luteum (CL) was calculated from the sum of different stages in the same ovary, including fresh CL or in regression and involution, according to Sandrock et al. (2009). Furthermore, the follicles were evaluated morphologically, according to Ferreira et al. (2018). Like Hu et al. (2004) and Ferreira et al. (2018), the data were expressed in percentage of follicles/mm^2 ovary. The histological evaluation of the duodenum was carried out based on previous studies by Mowat & Ferguson (1982), Zhang et al. (2024), Gałęcka et al. (2024), and Li et al. (2024), focusing on villus and crypt integrity, inflammatory cell influx, vacuolization and edema in the mucous layer and submucosal layer.

3.2.4 STATISTICAL ANALYSIS

Our study's statistical analyses were conducted using GraphPad Prism software Version 9.3.0 or the software package Paleontological Statistics PAST. Initially, the homogeneity of variances (assessed using the Bartlett test) and distribution of residual data (assessed using the Shapiro-Wilk test) were evaluated. Parametric data were compared via Student's t-test (Kim, 2015). When assumptions of normality and homogeneity of variance could not be met, the data were analyzed via the Mann-Whitney nonparametric test (McKnight & Najab, 2010). Significance levels were set at Type I error (p) values lower than 0.05. Furthermore, the data were subjected to correlation analysis, principal component analysis (PCA), and cluster analysis (CA). Pearson's correlation coefficient (r) value was calculated to identify relationships between toxicity biomarkers. In PCA and CA, the data were transformed using the log10 (x + 1) transformation to eliminate effects from differences in units before analysis, as recommended by Cao et al. (1999). Clustering was realized through CA, using the Euclidean distance and Ward's method, and the cophenetic correlation coefficient was calculated to evaluate the similarity between observations in the dendrogram produced by the clustering algorithm, according to Saraçli et al. (2013).

3.3 RESULTS

Our analyses revealed that ingested water contaminated with SARS-CoV-2 lysate protein did not affect water or feed intake nor alter body mass gain throughout the exposure period (Figures S1A-C – see "Apêndice 1"). Additionally, no clinical signs commonly associated with systemic toxicity, such as seizures, frothing at the mouth, piloerection, altered respiratory patterns, diarrhea, lethargy, abnormal postural adjustments, or mortality, were observed in either the treated or untreated groups. On the other hand, at the end of the experiment, the animals in the SARS-CoV-2-exposed group showed less locomotor activity (Figure 4A) and anxiety-like behavior, characterized by the predominance of thigmotaxis behavior in the OFT when compared to unexposed animals (Figure 4B). Regarding the NOR test, the object exploration rates obtained for the unexposed mice validated the test, demonstrating that the discrimination between the NO and FO was a consequence of the memory recall acquired and retained during the training session and evoked in the test section (Figures S2A-B - see “Apêndice 1”). However, the lower NOR indices (index 1 to 3) of mice exposed to SARS-CoV-2 lysate protein are suggested by memory deficits (Figures 4C-E). On the other hand, we did not observe differences between the experimental groups regarding the immobility time in the TST (Figure 4F), demonstrating that ingesting water containing SARS-CoV-2 lysate protein did not induce a depressive-like effect. Furthermore, the predictive biomarkers of mutagenicity (micronuclei index) (Figure S3A), genotoxicity (TL, Tail DNA%, and OTM) (Figures S3B-D – see “Apêndice 1”), and hematological changes (Figure S4 – see “Apêndice 1”) did not differ between animals unexposed and exposed to SARS-CoV-2 lysate protein.

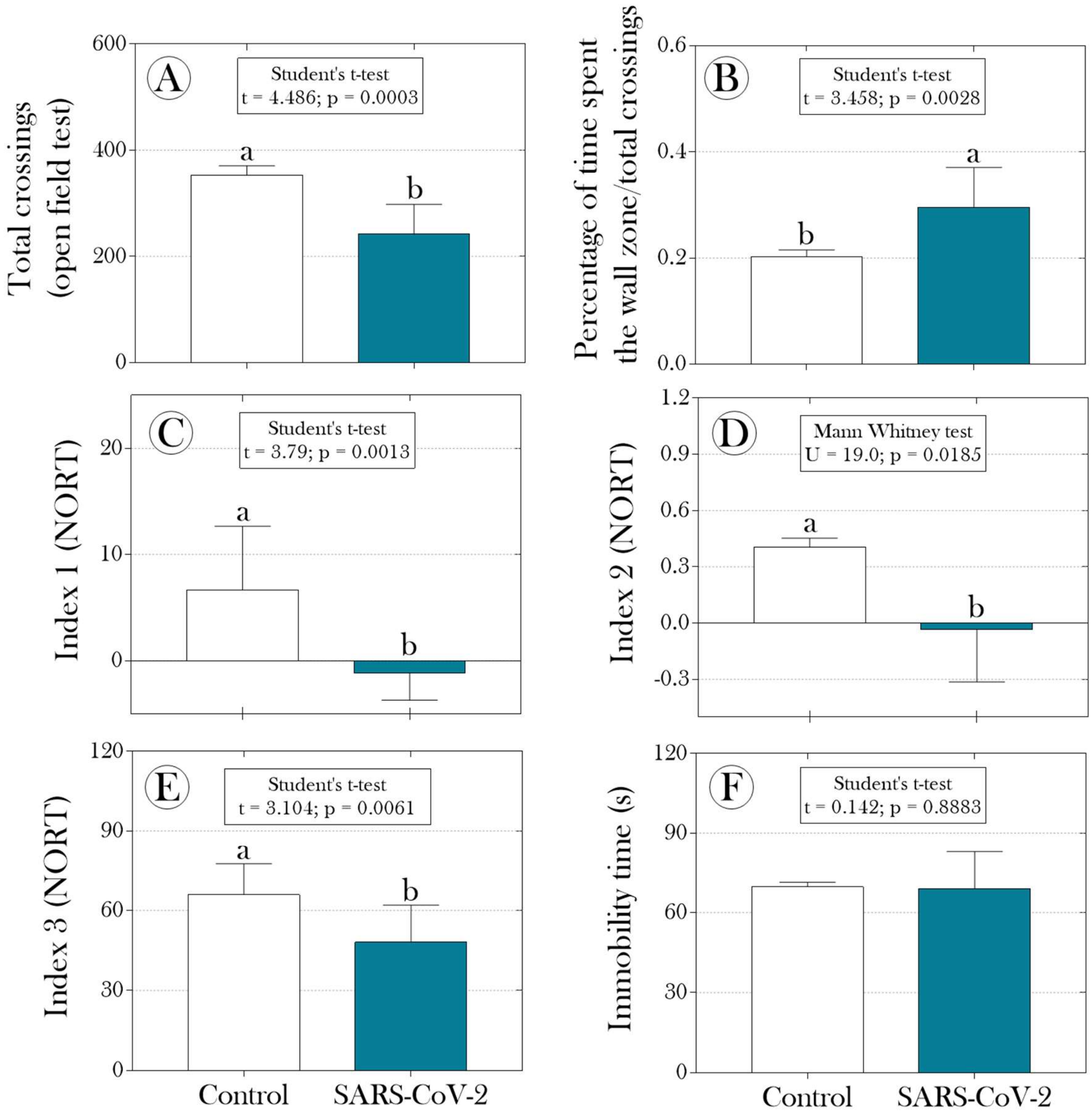

Figure 4. Behavioral responses of C57Bl/6J female mice unexposed ("control" group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) ("SARS-CoV-2" group) in different tests. (A) Total crossings and (B) percentage of time spent in the wall zone/total crossings in the open field test, (C-E) object recognition indices in the novel object recognition test (NORT), and (F) immobility time in the tail-suspension test. Parametric data (compared by the Student's t-test) are presented by the mean + standard deviation, whereas non-parametric data (compared by the Mann-Whitney test) are presented by the median and interquartile range. Statistical summaries are presented next to the graphs. Distinct lowercase letters indicate significant differences between experimental groups.

In serological assessments, we also did not notice differences between the groups in relation to albumin (Figure 5B), triglycerides (Figure 5C), total cholesterol (Figure 5D), and creatinine levels (Figure 5E). Furthermore, ingesting water containing SARS-CoV-2 lysate protein did not alter the activity of alpha-amylase, alkaline phosphatase, and gamma-glutamyl transferase (Figures 6A-C, respectively). However, the serum total protein levels (Figure 5A), bilirubin levels (Figure 5F), and lactate dehydrogenase activity (Figure 6D) differed significantly between the experimental groups. While the total protein level was 13.5% lower in the SARS-CoV-2-exposed group, the bilirubin levels and lactate dehydrogenase activity were 56.8% and 37.1% higher in these animals (respectively), when compared to the unexposed mice.

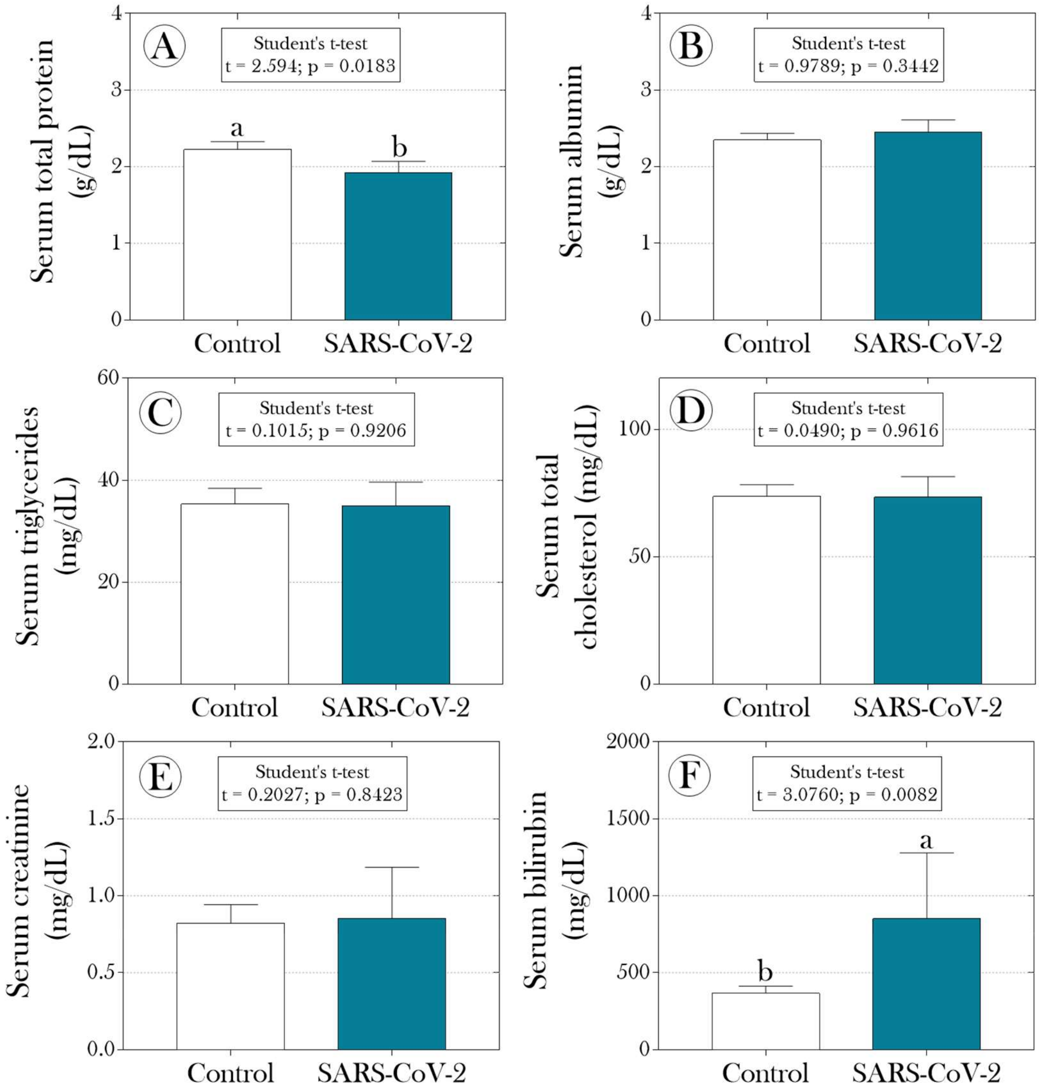

Figure 5. Serological biochemical biomarkers evaluated in C57Bl/6J female mice unexposed (“control” group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) (“SARS-CoV-2” group). (A) Total protein, (B) albumin, (C) triglycerides, (D) cholesterol, (E) creatinine, and (F) bilirubin levels. Parametric data (compared by the Student's t-test) are presented by the mean + standard deviation. Statistical summaries are presented next to the graphs. Distinct lowercase letters indicate significant differences between experimental groups.

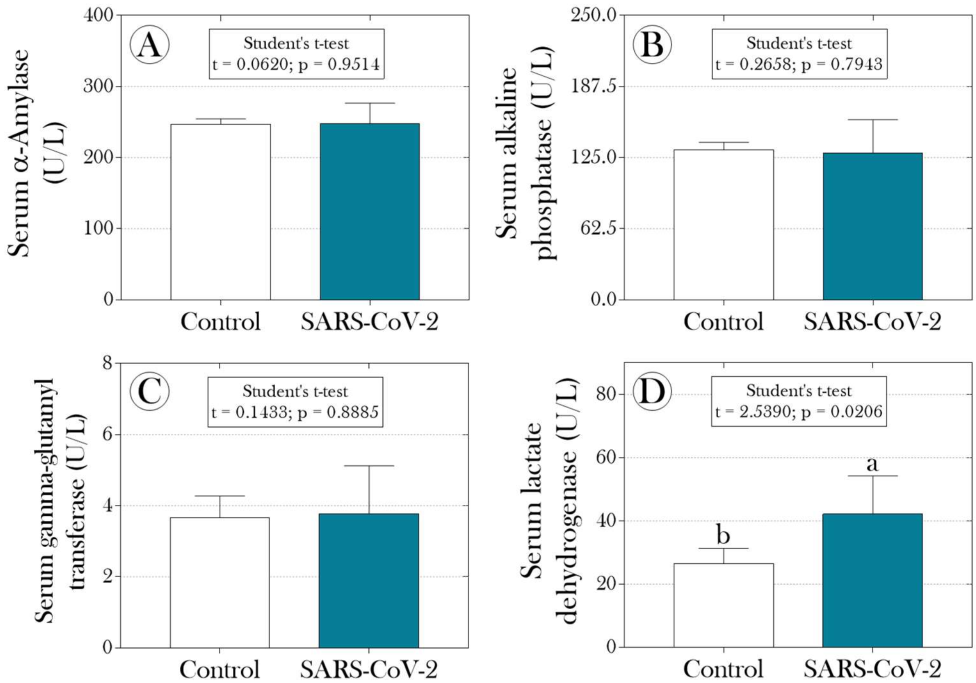

Figure 6. Serological enzyme activities evaluated in C57Bl/6J female mice unexposed ("control" group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) ("SARS-CoV-2" group). (A) Alpha-amylase, (B) alkaline phosphatase, (C) gamma-glutamyl transferase, and (D) lactate dehydrogenase. Parametric data (compared by the Student's t-test) are presented by the mean + standard deviation. Statistical summaries are presented next to the graphs. Distinct lowercase letters indicate significant differences between experimental groups.

Regarding tissue biochemical assessments, we noticed that dopamine levels (Figure 7A) and cholinesterase activity [AChE and BChE (Figure 7B)] in the brains of animals exposed to SARS-CoV-2 lysate protein were significantly correlated with ROS levels and their behavioral responses (Figure 7C). The increases in dopamine levels, AChE, and BChE activity in the SARS-CoV-2-exposed group were 17.5%, 18%, and 62.9% higher than in the unexposed mice (Figures 7A-B). In the brain, ROS production in animals that ingested water containing SARS-CoV-2 lysate protein was 16.8% higher than in unexposed animals (Figure 8A). In the duodenum and ovary, changes associated with treatment were characterized by greater ROS, MDA, and nitrite production (Figures 8A-C, respectively). Furthermore, greater SOD activity was observed in the brain, liver, and ovary of animals exposed to SARS-CoV-2 lysate protein (Figure 9A). On the other hand, the lower activity of SOD and catalase in the duodenum, as well as catalase activity in the brain of animals exposed to SARS-CoV-2, suggests that the treatment induced a suppression of antioxidant activity in these organs (Figure 9A).

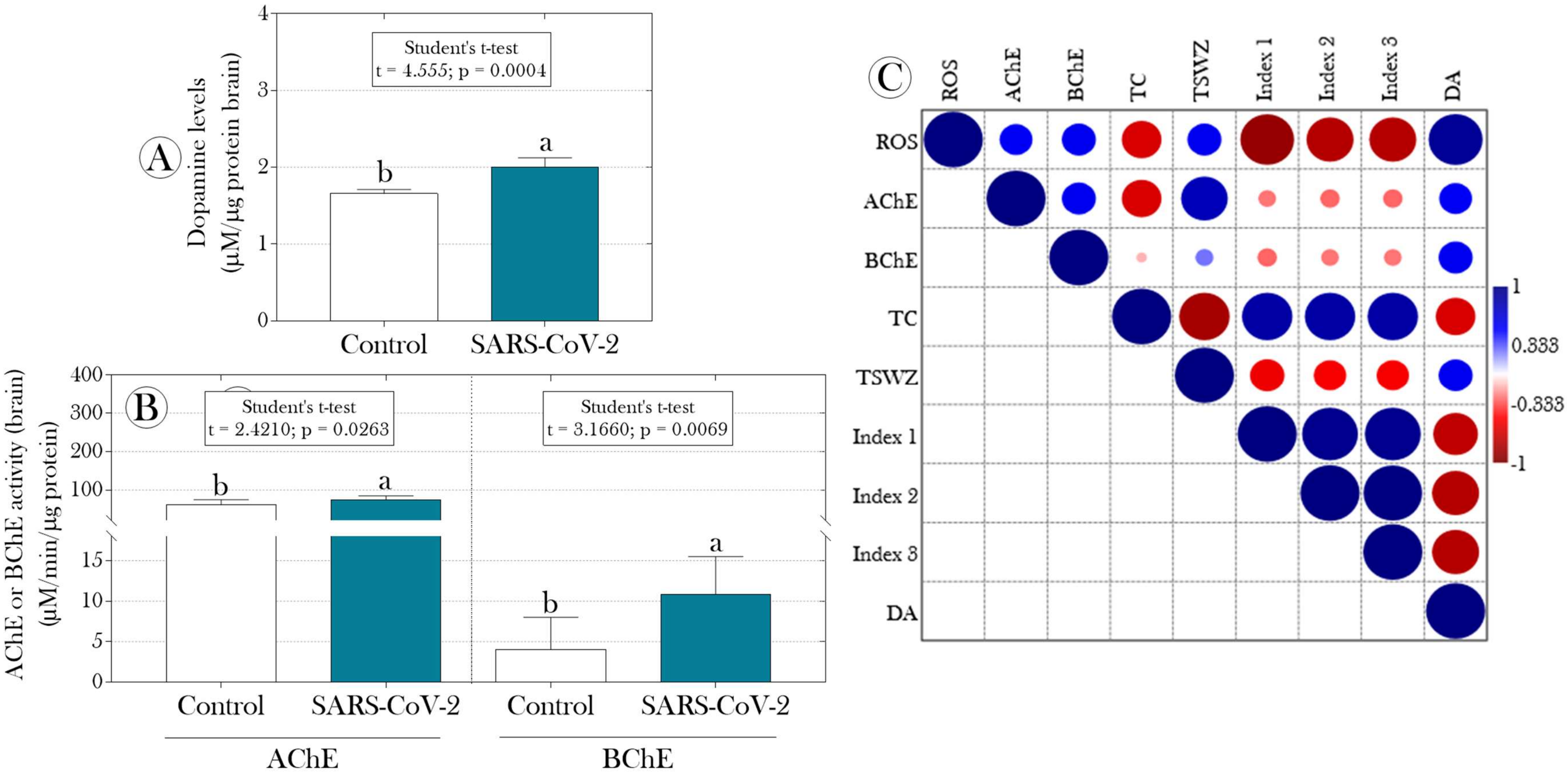

Figure 7. (A) Dopamine (DA) levels and (B) acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity in brain of C57Bl/6J female mice unexposed ("control" group) or exposed to SARS-CoV-2 lysate protein (by the oral, to 20 µg/L) ("SARS-CoV-2" group). (C) Correlation analysis between different biomarkers predictive of neurotoxicity. In "A" and "B," parametric data (compared by the Student's t-test) are presented by the mean + standard deviation. Statistical summaries are presented next to the graphs. Distinct lowercase letters indicate significant differences between experimental groups. ROS: reactive oxygen species, TC: total crossing, TSWZ: the ratio between time spent in the wall zone and total crossing, and index 1 to 3: object recognition indices (see Eq. 1 to 3 in section 2.3.1.2, in "M&M").

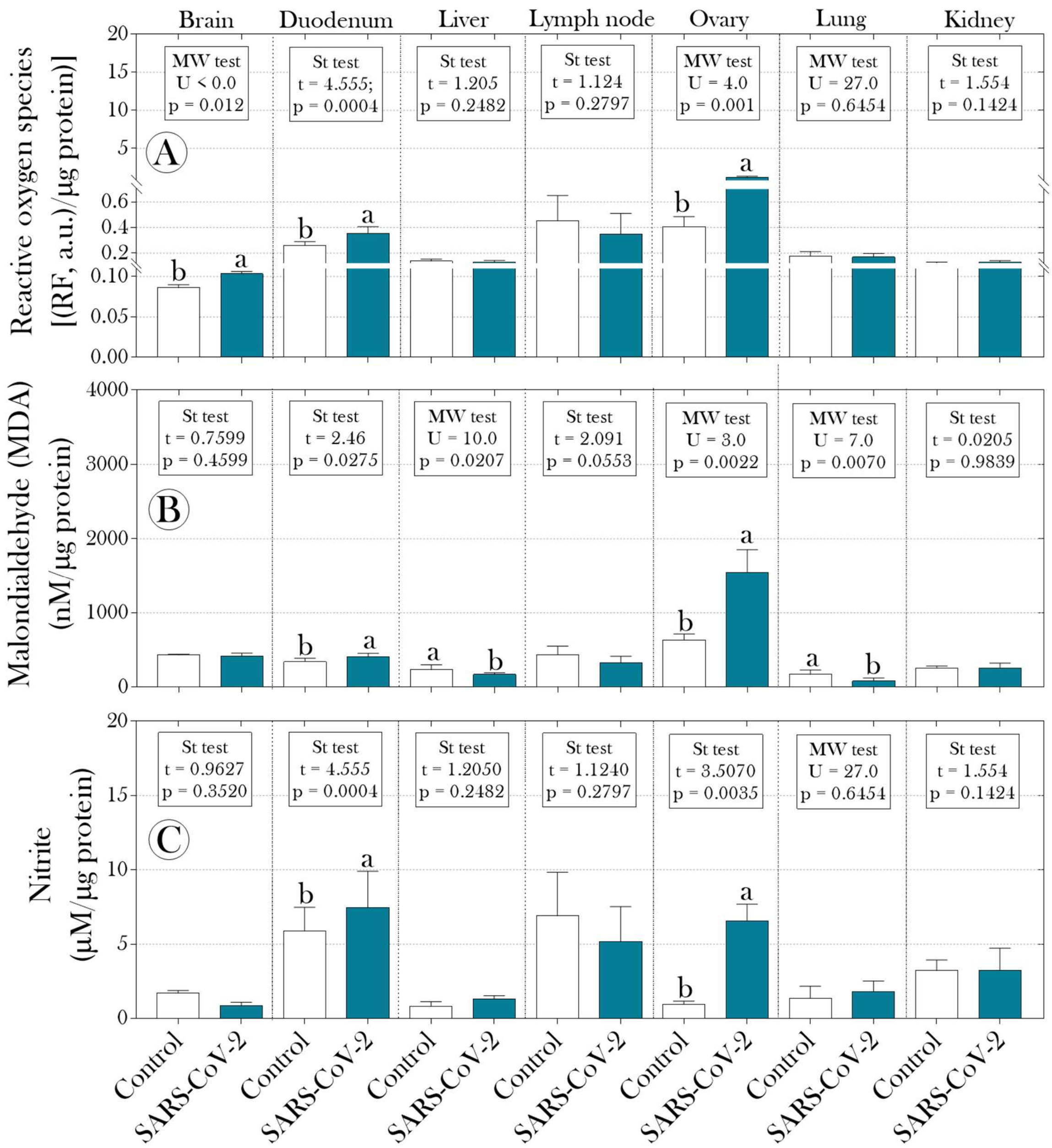

Figure 8. (A) Reactive oxygen species, (B) malondialdehyde, and (C) nitrite levels in different tissues/organs of C57Bl/6J female mice unexposed ("control" group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) ("SARS-CoV-2" group). Parametric data [compared by the Student's t-test (St test)] are presented by the mean + standard deviation, whereas non-parametric data [compared by the Mann-Whitney test (MW test)] are presented by the median and interquartile range. Statistical summaries are presented next to the graphs. Distinct lowercase letters indicate significant differences between experimental groups. RF: relative fluorescence and a.u.: arbitrary units.

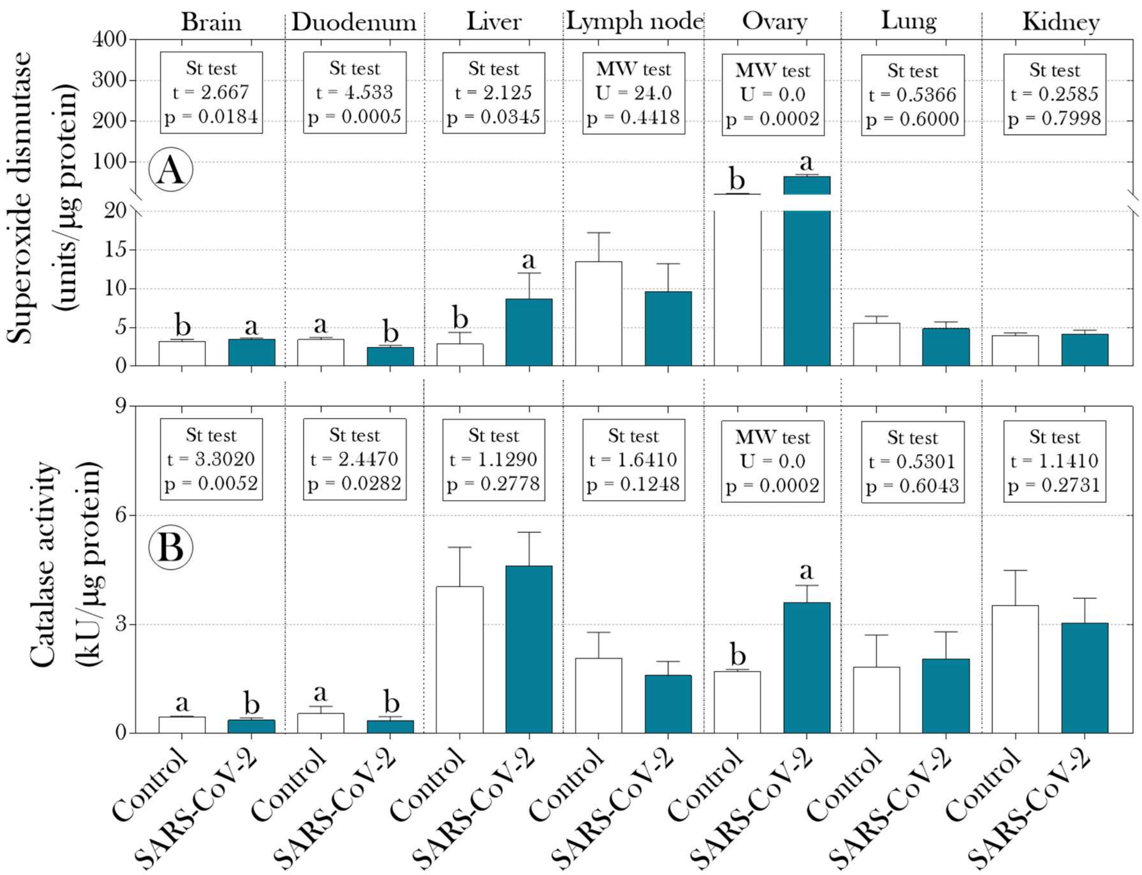

Figure 9. (A) Superoxide dismutase (SOD) and (B) catalase activity in different tissues/organs of C57Bl/6J female mice unexposed ("control" group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg /L) ("SARS-CoV-2" group). Parametric data [compared by the Student's t-test (St test)] are presented by the mean + standard deviation, whereas non-parametric data [compared by the Mann-Whitney test (MW test)] are presented by the median and interquartile range. Statistical summaries are presented next to the graphs. Distinct lowercase letters indicate significant differences between experimental groups.

To explore the possible relationship between the elevated ROS, MDA, and nitrite production in the duodenum and ovaries of animals exposed to SARS-CoV-2 to a possible induction of an inflammatory process, the levels of pro- and anti-inflammatory cytokines were evaluated. Figures 10A-B demonstrate that the IFN-γ and IL-10 levels were higher in the duodenum and ovary of animals from the SARS-CoV-2-exposed group, coinciding with the high ROS, MDA, and nitrite production in these same organs when compared to the "control" group. Regarding histological analyses, in Figure 11 it is possible to observe that the tissue structures of the duodenum of animals unexposed or exposed to SARS-CoV-2 remained predominantly preserved, with no hyperemia, degenerative changes in the tips of villi, loss of structural integrity of mucosal folds, degenerative mucosal epithelium (hypertrophy, vacuolation, hyperchromasia) necrosis, desquamation of mucosal epithelium, cellular debris, or excessive mucus in gut of lumen. However, in the SARS-CoV-2-exposed group, we noticed the presence of inflammatory infiltration, especially in the submucosa (Figure 11J) and in the lamina propria (Figures 11H and 11K), as well as a greater thickness of the muscularis externa, which consists of two-layer muscles: inner circular and outer longitudinal (Figures 11H-I).

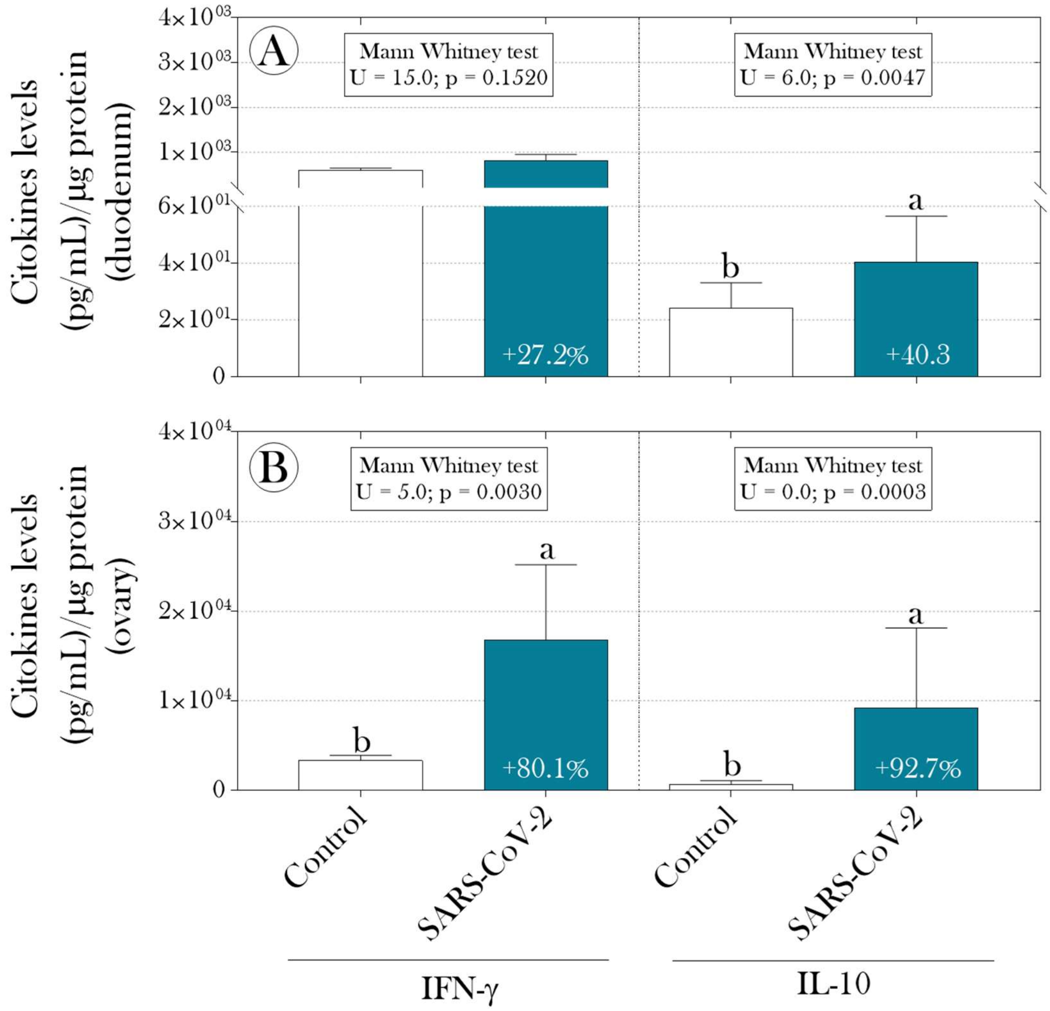

Figure 10. Interferon-gamma (IFN-γ) and interleukin-10 (IL-10) levels in the (A) duodenum and (B) ovary of C57Bl/6J female mice unexposed ("control" group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) ("SARS-CoV-2" group). Non-parametric data [compared by the Mann-Whitney test (MW test)] are presented by the median and interquartile range. Statistical summaries are presented next to the graphs. Distinct lowercase letters indicate significant differences between experimental groups.

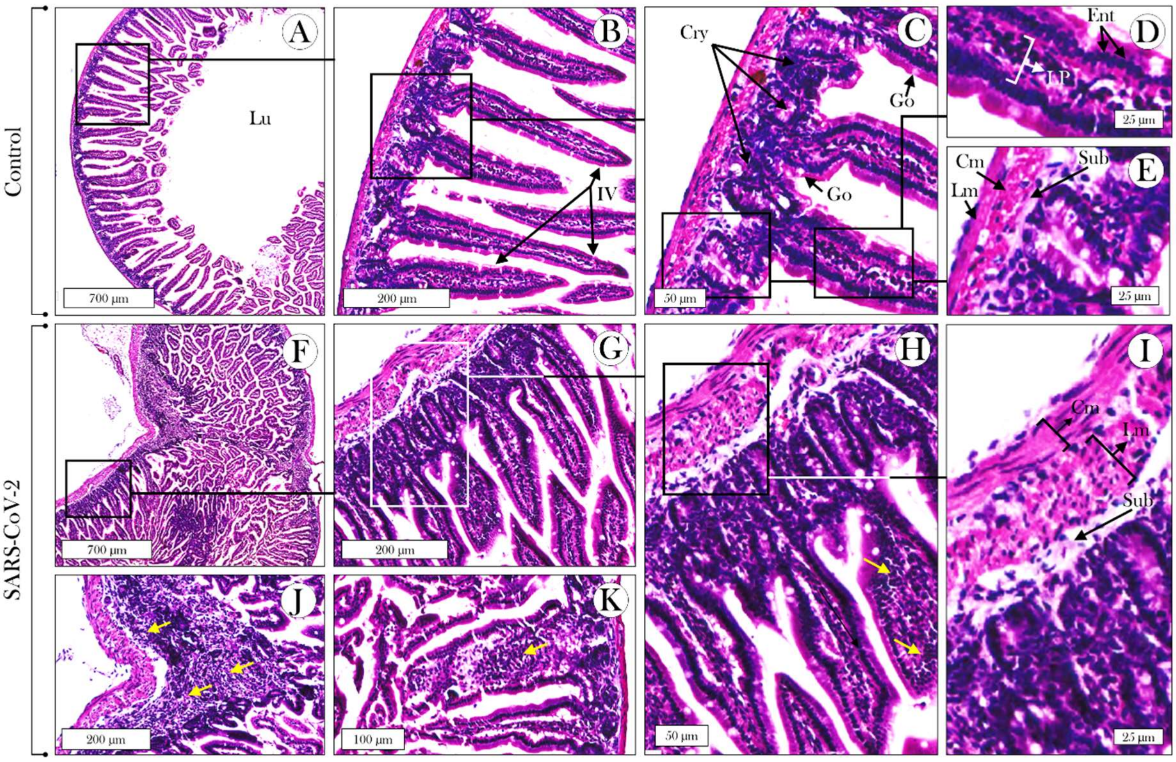

Figure 11. Representative photomicrographs of H&E-stained duodenal tissue sections of C57Bl/6J female mice unexposed ("control" group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) ("SARS-CoV-2" group). (A-E) Control and (F-K) SARS-CoV-2-exposed group. Lu: intestinal lumen; IV: intestinal villi; Cry: crypts; Go: goblet cells; Ent: enterocytes; Cm: circular muscle; Lm: longitudinal muscle; LP: own lamina; Sb: submucosa. In "H," "J," and "K," yellow arrows indicate the presence of inflammatory infiltrates.

In the histological analysis of the ovary, we noticed that mice unexposed and exposed to SARS-CoV-2 lysate protein presented follicles at all stages of development, from the primordial through the antral follicle stage, as well as the presence of corpora lutea (Figures 12A-F). Furthermore, the "control" group revealed a normal ovarian histological structure with an ovarian cortex characterized by multiple follicles in various stages of development, an outer simple cubical epithelium, and a central medulla made of the vascularized ovarian stroma (Figure 13A). However, quantitatively, the number of primary follicles was lower in the ovaries of animals exposed to SARS-CoV-2, corresponding to only 39.5% of the frequency of these follicles in unexposed mice (Figure 12G). Furthermore, the SARS-CoV-2-exposed group showed disrupted follicular structure (Figure 13B1), multiple vacuoles between the granulosa cells (Figures 13C-D), desquamated granulosa cells into follicular antrum (Figures 13E), dilated and congested vessels in the ovarian medulla (Figure 13F), hemorrhagic event (Figure 13B2) and corpus luteum cells with hydropic degeneration and vacuoles (Figure 13G), and frequency of degenerating follicles (atresic) (Figures 13H-K), when compared to control (Figure 13A).

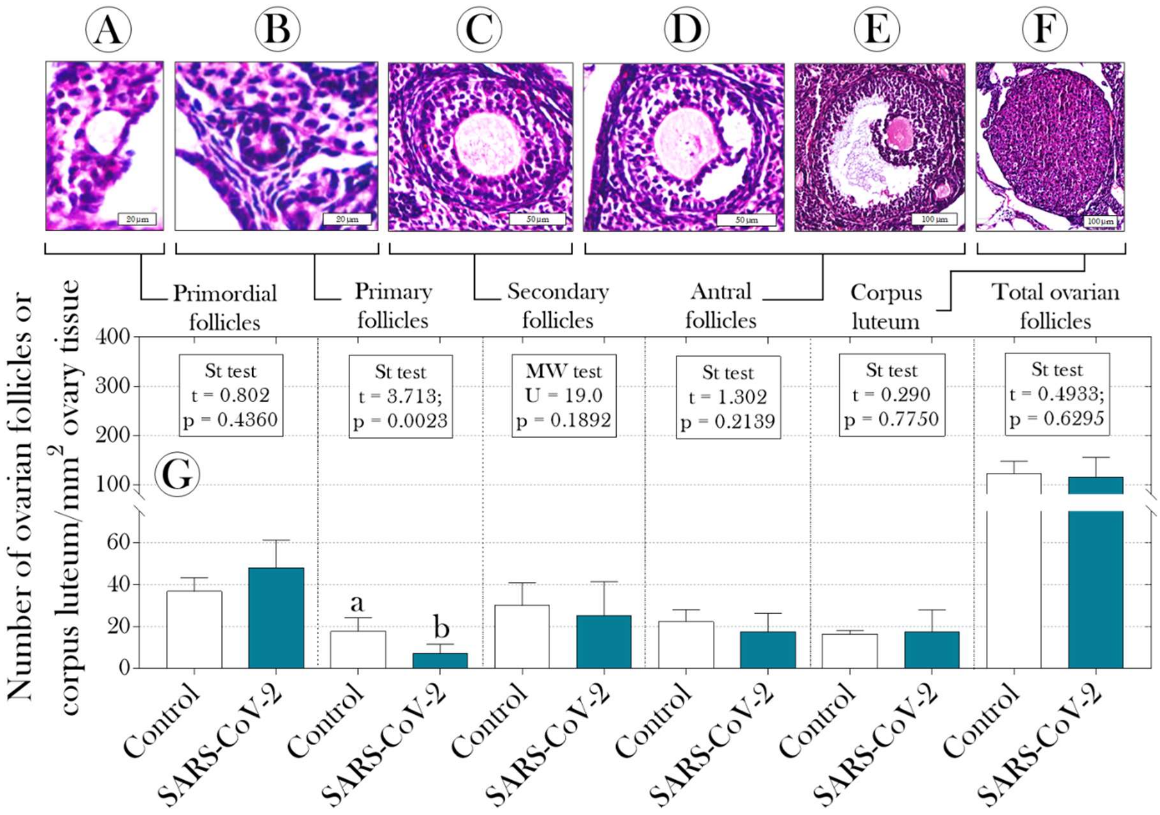

Figure 12. (A-F) Representative photomicrographs of H&E-stained ovarian tissue sections demonstrating follicles at distinct stages of development and (G) number of ovarian follicles or corpus luteum/mm2 tissue of C57Bl/6J female mice unexposed ("control" group) or exposed to SARS -CoV-2 lysate protein (orally, to 20 µg/L) ("SARS-CoV-2" group). In "G," parametric data [compared by the Student's t-test (St test)] are presented by the mean + standard deviation, whereas non-parametric data [compared by the Mann-Whitney test (MW test)] are presented by the median and interquartile range. Statistical summaries are presented next to the graphs. Distinct lowercase letters indicate significant differences between experimental groups.

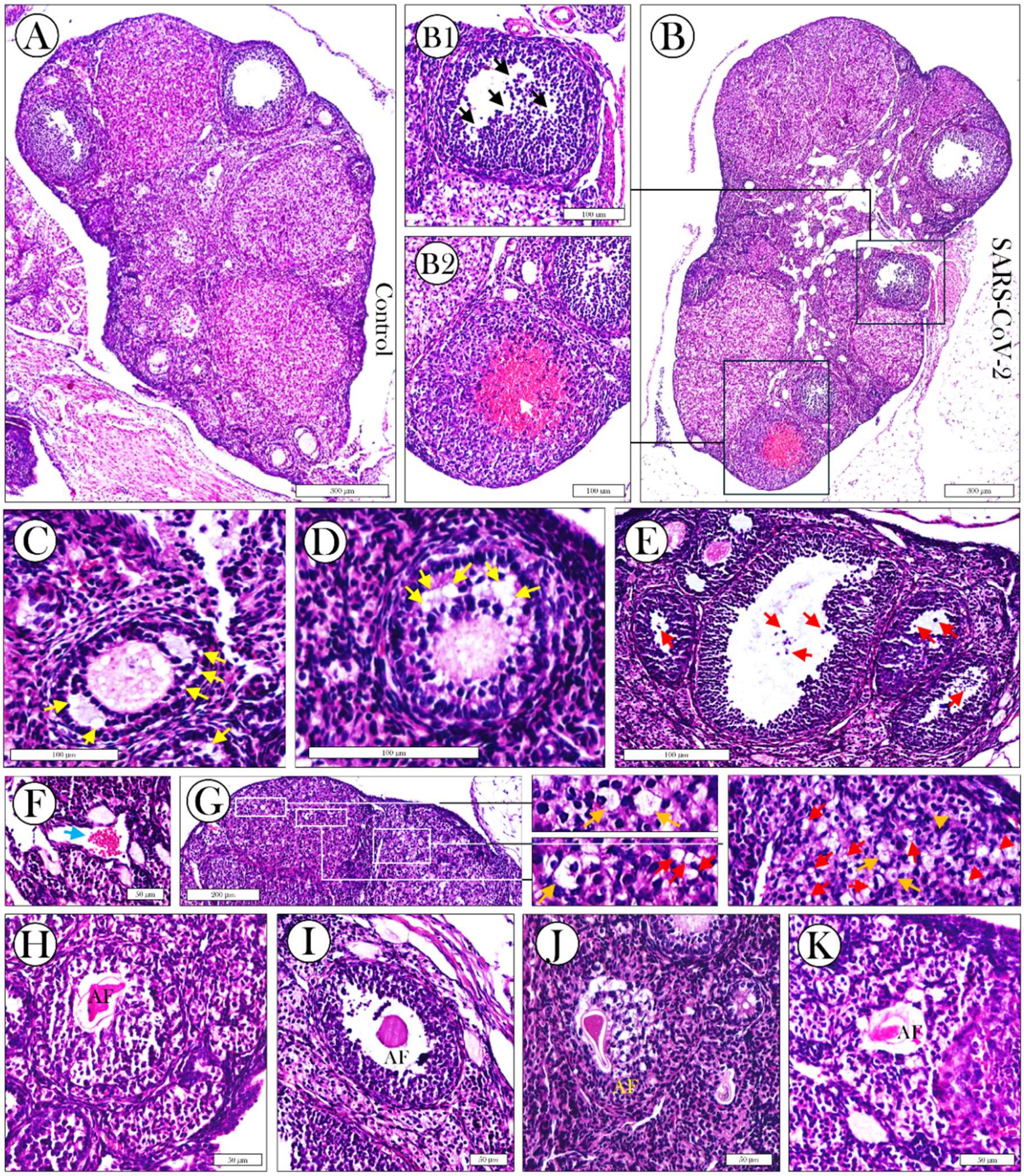

Figure 13. Representative photomicrographs of H&E-stained ovarian tissue sections of C57Bl/6J female mice unexposed ("control" group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) ("SARS-CoV-2" group). (A) Control and (B-K) SARS-CoV-2-exposed group. In "B1", black arrows indicate disrupted follicular structure; in "B2", the white arrow indicates a hemorrhagic event in the corpus luteum; in "C-D," yellow arrows indicate multiple vacuoles between granulosa cells; in "E," red arrows indicate desquamated granulosa cells into follicular antrum; in "F," the blue arrow indicates dilated and congested vessel in the ovarian medulla; in "G," the orange and red arrows indicate corpus luteum cells with hydropic degeneration and vacuoles, respectively; in "H to K," atretic follicles (AF) are presented, which are ovarian follicles that began to mature but did not become the dominant follicle and have involuted.

To assess the overall response of animals to treatments, minimize data complexity, and more clearly identify SARS-CoV-2 lysate protein toxicity, we analyzed the PCA and CA using a database of 80 biomarkers (Table S1 – see "Apêndice 1"). In PCA, we showed that the three main components (PCs) cumulatively explained 60.95% of the total variation (PC1: 42.729%, PC2: 10.725%, and PC3: 7.487%), with PC-eigenvalues > 5.0 (Figure S5A – see "Apêndice

1”). The variables with the largest loading in PC1 were (i) ROS, MDA, nitrite, IFN-γ/IL-10 levels and SOD and catalase activity in the ovary; (ii) IFN-γ/IL-10 production and SOD activity in the duodenum; (iii) BChE activity in the brain; (iv) bilirubin levels and lactate dehydrogenase activity in the serum; and (v) number of primary follicles in the ovary (Figure S5B - see “Apêndice 1”). Furthermore, Figures 14A-D demonstrate that the groups were clearly separated when the different PCs were plotted (PC1 *versus* PC2, PC1 *versus* PC3, and PC1 *versus* PC2 *versus* PC3). While the “control” group was positioned in negative quadrants of PC1, the group formed by mice exposed to SARS-CoV-2 lysate protein was positioned in positive quadrants of the same PC. Such results were consistent with the heat map clustering for evaluated endpoints (Figure 15). In heat–map, it is possible to observe that the variables were horizontally clustered in two clusters (cluster 1: “control” group and cluster 2: “SARS-CoV-2” group; cophenetic correlation coefficient = 0.7921), confirming that the variables that presented higher loading in PC1 showing the largest changes upon SARS-CoV-2 exposure.

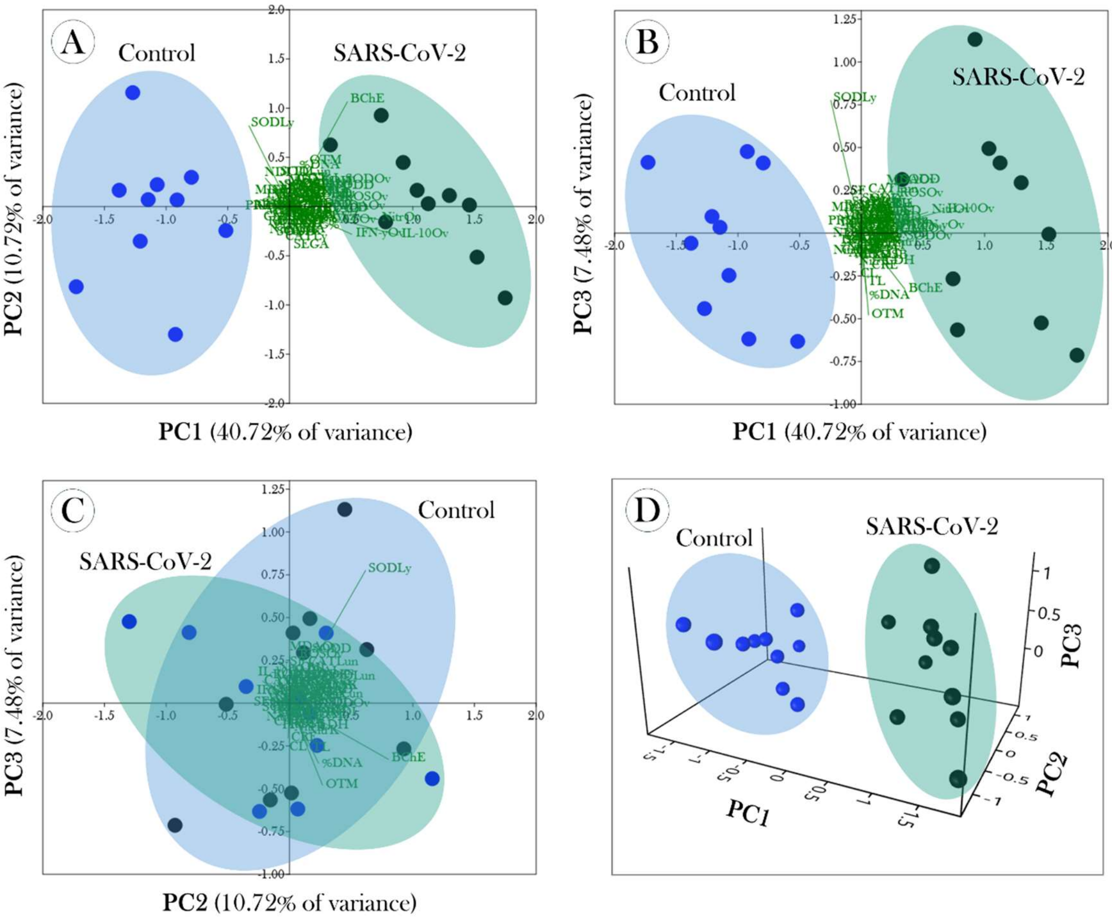

Figure 14. Biplot of the loadings and scores obtained in the principal components analysis (PCA), using different toxicity biomarkers evaluated in C57Bl/6J female mice unexposed (“control” group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) (“SARS-CoV-2” group). (A) Biplot of PC1 versus PC2; (B) PC1 versus PC3; (C) PC2 versus PC3; and (D) three-dimensional score plot (PC1 versus PC2 versus PC3). The

blue and black circles represent the scores of the “control” and “SARS-CoV-2” groups, respectively, extracted from PCA. The green lines in “A to C” indicate the PC loading vectors. Groups have been clustered using ellipses for clarity (blue: “control” group and “green: “SARS-CoV-2” group). PC: main component. The abbreviations' meanings in “A to C” are presented in Table S1 – see “Apêndice 1.”

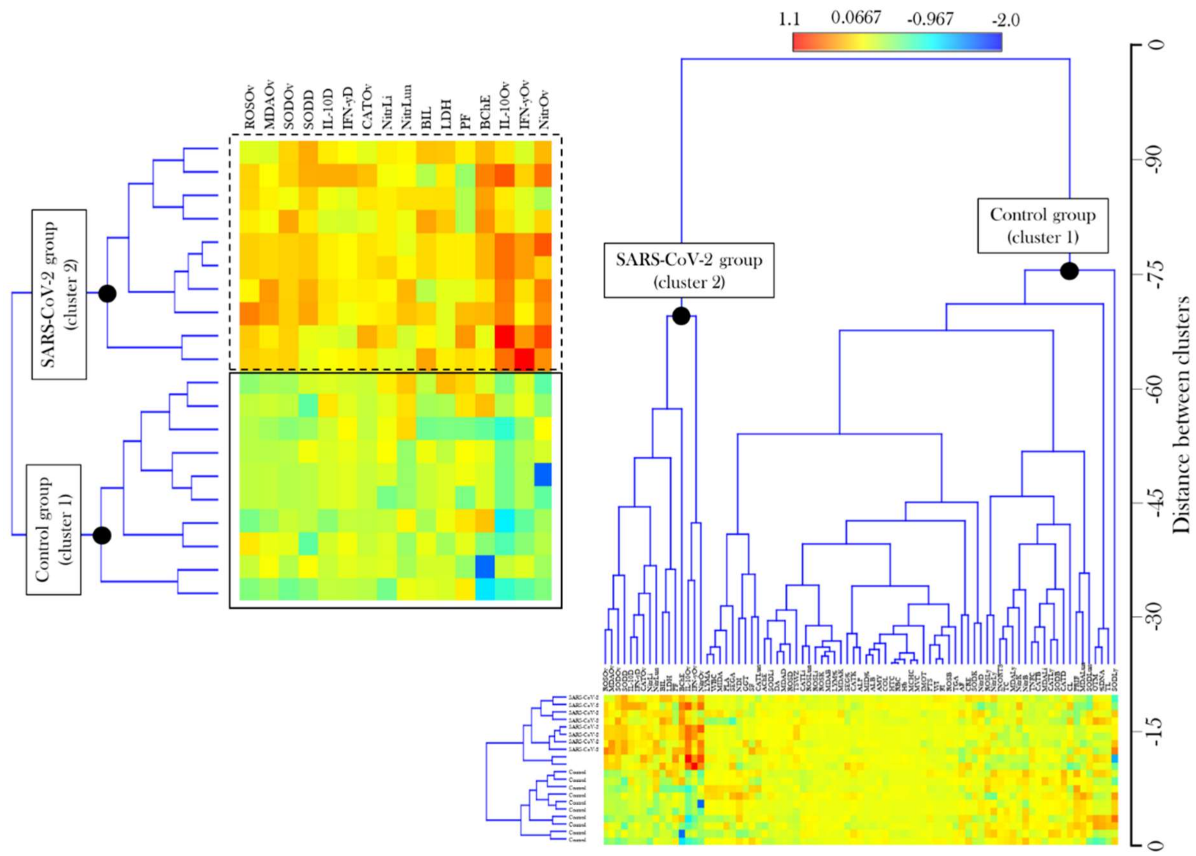

Figure 15. Heat maps showing results of the tree clustering (Ward's method, Euclidean distances) of the toxicity biomarkers evaluated in C57Bl/6J female mice unexposed (“control” group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) (“SARS-CoV-2” group). The abbreviations' meanings in “A to C” are presented in Table S1 – see “Apêndice 1.”

3.4 DISCUSSION

Arguably, the COVID-19 pandemic is considered the most significant human threat since the Second World War and the greatest global health disaster of the 21st century. Its impacts have permeated different areas and sectors of society, and outbreaks, endemics, and epidemics of COVID-19 are not likely to go extinct shortly; continuous measures and actions are necessary to prevent the spread of the infection caused by SARS-CoV-2 and prevent a new pandemic scenario from being established. In addition to human health, attention must also be given to the impacts of the pandemic on natural ecosystems and biota to expand our knowledge about the real magnitude of the effects of the COVID-19 pandemic on the environment and contribute to managing its potential risks. Although a wide range of studies has already associated the COVID-19 pandemic with the increase in biomedical and municipal solid waste generation (Olabi et al., 2024; Wan et al., 2024), haphazard disposal of safety equipment (Najafighodousi et al., 2024), reduction of recycling (Ebner & Iacovidou, 2021; Inamdar, 2022), and extensive use of disinfectants (Rume & Islam, 2020), the consequences of the dispersion of SARS-CoV-2 in aquatic environments are still little known. As discussed by Malafaia et al. (2024), evaluating the impacts of the interaction of non-target organisms with non-chemical toxic agents (e.g., viruses, fungi, and/or bacteria) is essential when planning strategies/actions aimed at protecting and/or mitigating the effects of human pathogens on non-target species. In this sense, our study is in line with previous studies that demonstrated that the interaction of non-target organisms with SARS-CoV-2 or its fragments affects the health of different aquatic species and sheds light on the health risks to mammals (not humans) associated with the ingestion of water containing SARS-CoV-2 lysate protein. From the evaluation of several biomarkers, we observed that although mutagenic, genotoxic, and hematological effects were not evidenced, oral exposure of C57Bl/6J female mice to SARS-CoV-2 induced behavioral, serological, and biochemical changes that, together, demonstrate that the introduction of these viruses into aquatic environments also represents a risk to the health of terrestrial organisms.

Initially, our data suggest that the behavioral changes induced by ingestion of water containing SARS-CoV-2 lysate protein [i.e., hypoactivity, anxiety-like behavior, and memory deficit (Figures 4D-E)] were possibly resulting from changes in dopaminergic and cholinesterasic balance, characterized by a significant increase in dopamine production (Figure 7A) and AChE and BChE activity (Figure 7B). While AChE (true cholinesterase) and BChE (a nonspecific cholinesterase enzyme) play an essential role in mediating neurotransmission in cholinergic synapses, which control the higher brain functions (Tsim & Soreq, 2013; Li et al., 2021; Harrington et al., 2022); the dopaminergic system plays important roles in neuromodulation, such as locomotor control, motivation, reward, and cognitive function (Juárez-Olguín et al., 2016; Klein et al., 2019). Previous studies, such as those by Trang & Khandhar (2019), reinforce our hypothesis by demonstrating that the increase in cholinesterases culminates in the reduction of neuronal transmission and signaling between synapses and, consequently, in the reduction of locomotor activity (Trang & Khandhar, 2019), predicting greater levels of anxiety (Mooney-Leber et al., 2021). Furthermore, it has already been demonstrated that increased DA neurotransmission causes hypoactivity in previously hyperactive mice when evaluated in the OFT (Avale et al., 2004; Hagino et al., 2015;

Yamaguchi et al., 2017), which the dopamine levels and its metabolites are associated with anxiety-like behavior (Zarrindast & Khakpai, 2015) and that dopaminergic signaling has a significant role in regulating NOR test behavior (Belcher et al., 2008; Camarasa et al., 2010; Yang et al., 2017).

However, in addition to being very limited, previous literature on the effects of exposure of non-target organisms to SARS-CoV-2 or its fragments has demonstrated that behavioral impacts depend on several factors, including the species/model organism, the age/developmental stage of the animals, the routes and times of exposure, and the type of material used in the exposures (fragments or inactivated viruses) and their respective concentrations. In the study by Luz et al. (2023), after 24 h of intraperitoneal administration of PSPD-2002 (peptide fragments of the SARS-CoV-2 Spike protein), the mice did not present hypo/hyperactivity or anxiety-like behavior (in the OFT), nor depressive-like behavior in the forced swimming test. Mendonça-Gomes et al. (2021) demonstrated that aquatic exposure to PSPD-2002 and PSPD-2003 (up to 40 μg/L), for 48 h, induced hyperactivity in *Culex quinquefasciatus* larvae and, in fish (*Poecilia reticulata*), Malafaia et al. (2022) demonstrated that although habituation learning was not altered by the peptides (40 μg/L, 35 days exposure), anxiety-like behavior was observed. On the other hand, when *P. reticulata* were exposed to SARS-CoV-2 lysate protein (the same one used in our study), the animals showed locomotor changes (inferred by the lower number of quadrant crossings, distance covered, and swimming speed) and higher anxiety index in the OFT (Ferreira et al., 2023). However, in adult zebrafish (*Danio rerio*), exposure to SARS-CoV-2 lysate protein negatively affected habituation memory and induced deficits in response to potential aquatic predator (*Geophagus brasiliensis*), with no locomotor alterations or anxiety/anxiolytic-like behavior (Luz et al., 2023).

In our study, in particular, changes in dopamine production and cholinesterase activity may be associated with several factors that need further investigation. The significant correlation between ROS production in the brain and the increase in these neurological biomarkers strongly suggests that oxidative stress constitutes a possible cause of the changes. It is known, for example, that increased dopamine levels are an important source of oxidative stress in the brain since its degradation generates different ROS (Meiser et al., 2013). On the other hand, increased ROS production can impair dopamine recycling (Miyazaki & Asanuma, 2008), as well as affect dopamine transporters (Berman et al., 1996), considered key proteins responsible for regulating the homeostasis of this neurotransmitter (Masoud et al., 2015). Both situations can contribute to the maintenance of high levels of brain dopamine, and therefore, future investigations will be important to understand whether the increase in dopamine in animals exposed to SARS-CoV-2 lysate protein is due to an increase in ROS production or vice-versa. On the other hand, the increase in cholinesterase activity may, e.g., have been motivated by the increase in the synthesis and release of ACh in synaptic clefts after activation of the cholinergic anti-inflammatory pathway by SARS-CoV-2 fragments translocated to the nervous system central. As highlighted by Miao et al. (2013), ACh can act to inhibit mitochondria-, xanthine oxidase- and NAPDH oxidase-derived ROS formation and, thus, protect cells against oxidative stress. BChE, in particular, serves as an inherent protector from damage caused by toxic compounds before they reach AChE in synapses (Jasiecki et al., 2021). Therefore, the increase in its activity in the brains of animals of the SARS-CoV-2-exposed group may be related to its detoxification functions. In this context, future studies focusing on the identification of biological alterations related to the dopaminergic and cholinergic systems during the exposure of non-target organisms to the SARS-CoV-2 lysate protein may yield important insights about the neurochemical and molecular targets involved in neurotoxicity promoted by viral fragments.

In our study, we also noted that the biochemical effects of drinking water containing SARS-CoV-2 were organ-dependent. The absence of changes in the biomarkers assessed in lymph nodes, kidneys, and lungs (Figures 8 and 9) confirms that the ingestion of SARS-CoV-2 lysate protein does not trigger, in these organs, responses similar to those observed in humans infected with SARS-CoV-2 (Wang et al., 2021a; Abdullaev et al., 2021; Anastasio et al., 2021; Lins & Smaniotto, 2021; Halawa et al., 2022; Jansen et al., 2022; Zhang et al., 2023). On the other hand, the hyperbilirubinemia and increased LDH activity identified in the SARS-CoV-2-exposed group (Figures 5F and 6D, respectively) suggest hepatotoxicity induced by viral fragments. In addition to bilirubin (the main product resulting from the metabolism of the heme fraction of hemoglobin) being eliminated mainly in the liver (Hamoud et al., 2018), liver injury impacts the uptake of indirect bilirubin from plasma (by hepatocytes) and decreases direct bilirubin transport and clearance through the bile ducts (Guerra-Ruiz et al., 2021). The increased LDH production by hepatocytes in situations of acute liver failure (Kotoh et al., 2011) also corroborates our hypothesis. Another hypothesis is that bilirubin levels may have been elevated in the exposed group as a result of the antioxidant action that was taking place in the ovaries and intestines of these animals. According to Pisoschi et al. (2021), bilirubin has an inhibitory impact on the oxidizing activity of ROS, especially on the activity of NADPH oxidase. This is due to the antioxidant activity of heme oxygenase, the enzyme responsible for breaking down heme into biliverdin, free iron, and carbon monoxide, with biliverdin being rapidly reduced into bilirubin by biliverdin reductase (Pisoschi et al., 2021). This is a process that can be reversed and regenerated since inducible heme oxygenase-1 can be activated under conditions of high ROS levels caused by NADPH oxidase, resulting in greater bilirubin production and relief from oxidative stress (Datla et al., 2007).

Our data also suggest that the passage of SARS-CoV-2 through the gastrointestinal system triggers a response indicative of intestinal toxicity, which complements the recent study by Li et al. (2024), in which an intense inflammatory process was observed in the duodenum of mice inoculated intraperitoneally with SARS-CoV-2 Spike protein. Furthermore, the redox imbalance (Figures 8 and 9), elevated nitrite and IFN-γ production (Figures 8C and 10A, respectively), and the identification of inflammatory infiltration in the submucosa and lamina propria of the duodenum of animals of SARS-CoV-2- exposed group (Figure 11) coincide with reports of duodenitis in COVID-19 patients, presumably associated with a direct infection of the duodenal enterocytes by SARS-CoV-2 (Neuberger et al., 2022). Therefore, such changes constitute a common endpoint in SARS-CoV-2 pathogenesis in target (human) and non-target organisms (mice). It is essential that future studies evaluate whether the duodenal changes evidenced in our study are, as in infected humans, associated, e.g., with immune-mediated tissue injury and/or gut dysbiosis caused by microbiota (Wang et al., 2021b).

On the other hand, our findings point to negative and prominent impacts on the ovary of C57Bl/6J female mice exposed to SARS-CoV-2 lysate protein, which, to date, has not yet been reported in the female reproductive system in COVID-19 patients (Zupin et al., 2020; Li et al., 2020; Markiewicz-Gospodarek et al., 2021). In line with our observations, Fernandes et al. (2022) observed that female zebrafish injected with fragments 16 to 165 (i.e., the N-terminal portion of the SARS-CoV-2 Spike protein), after seven days, showed damage in the ovary, including extracellular disorganization, high frequency of follicular atresia and cellular infiltration. In our study, in particular, the inflammatory reactions triggered by fragments of SARS-CoV-2 translocated to the ovaries of animals may be the cause of oxidative and nitrosative stress (Figures 8A and 8C, respectively), as well as for the increase of IFN-γ (Figure 10B), whose high levels of IL-10 (anti-inflammatory cytokine) (Figure 10B) appear not to have been sufficient to reduce the production of the pro-inflammatory cytokine evaluated. However, the inference about the impact of these changes on the ovarian function of female mice exposed to SARS-CoV-2 in our study was restricted to the reduction in the number of primary follicles (Figure 12G), which motivates continued investigations to expand our knowledge about the possible impacts of drinking water containing SARS-CoV-2 lysate protein on oocyte quality and reproductive outcome.

Finally, it is important to highlight that our study is far from exhausting the varied investigative possibilities necessary to understand the ecotoxicological effects of ingesting water containing SARS-CoV-2 lysate protein by terrestrial organisms that are not targets of the infection. Although we used multiple biomarkers in our study, more specific approaches are needed to understand the mechanisms underlying the reported responses. Experimental designs that test the toxicity of SARS-CoV-2 lysate proteins in other experimental models, in different concentrations and exposure times, and with a focus on neurochemical and molecular targets, will be important to reveal other endpoints that can confirm the suggested ecological risks in our study. Equally important will be to evaluate whether biological homeostasis can be re-established after cessation of exposure to SARS-CoV-2, which will provide additional support for planning impact mitigation strategies.

3.5 CONCLUSION

In conclusion, our study did not confirm the presumption that exposure to SARS-CoV-2 lysate protein could induce mutagenic, genotoxic, and hematological effects. However, our study partially confirms the initial hypothesis by demonstrating that the ingestion of water containing SARS-CoV-2 lysate protein by C57Bl/6J female mice induces changes suggestive of behavioral disorders, imbalance in the homeostasis of the dopaminergic and cholinesterase systems, liver damage, as well as such as oxidative and nitrosative stress, inflammation, and duodenal/ovarian histopathological changes. Thus, our study advances knowledge about the ecotoxicological risks associated with the dispersion of SARS-CoV-2 in aquatic environments, provides new insights into the toxicity mechanisms of SARS-CoV-2 lysate protein in a non-target terrestrial organism of infection, and, therefore, expands our understanding of the real extent of the ecological/environmental impact of the COVID-19 pandemic.

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4 PRE-MATING AND GESTATIONAL TRADE-OFFS: REPRODUCTIVE PERFORMANCE, MATERNAL BEHAVIOR, AND ORGAN-SPECIFIC EFFECTS IN FEMALE MICE EXPOSED TO SARS-CoV-2 LYSATE PROTEIN

ABSTRACT

The rapid introduction of new environmental contaminants, including viral agents like SARS-CoV-2, has raised concerns about their indirect effects on non-target terrestrial organisms, especially through contaminated water sources. In this study, we investigated the potential impact of pre-mating and gestational exposure to SARS-CoV-2 lysate protein. We exposed C57Bl/6J female mice to a concentration of 20 µg/L for 30 days, administered via their drinking water. Contrary to our initial hypothesis, no significant changes were observed in gestational duration, litter size, or offspring biomass, indicating that reproductive parameters were not adversely affected. However, we confirmed that exposure resulted in significant neurobehavioral alterations, including increased anxiety-like behavior in the open field test and changes in maternal care, associated with elevated dopamine levels and altered cholinesterase activity in key regions of the central nervous system. Additionally, oxidative and nitrosative stress markers (ROS and NO) were elevated in mammary and ovarian tissues, suggesting a persistent inflammatory response. Therefore, our study demonstrates that even after the cessation of exposure, the observed neurochemical and behavioral changes may have long-lasting effects that, in natural environments, could represent a significant risk to population survival and stability, mainly through the modulation of neurochemical pathways and stress responses in non-target species.

Keywords: SARS-CoV-2. Maternal behavior. Neurochemical alterations. Oxidative stress. Reproductive toxicity. Terrestrial mammals.

4.1 INTRODUCTION

Natural environments are changing rapidly, which imposes on organisms the challenge of adapting to new environmental pressures quickly (Svenning et al., 2024). The introduction of new environmental contaminants, whether chemical or biological, further exacerbates the anthropogenic impacts already observed, resulting in adverse effects on the fitness of individuals and the dynamics of their populations, triggering maladaptive responses (Cheng et al., 2024; Rathod et al., 2025). In this scenario, the COVID-19 pandemic, caused by SARS-CoV-2, has not only brought severe consequences for humans in biomedical, epidemiological, social, economic, and political terms (Miyah et al., 2022; Naseer et al., 2023; Khan et al., 2024), but has also raised concerns about the indirect effects of this pathogen on non-target organisms, especially aquatic ones, as reported in the studies by Mendonça-Gomes et al. (2021), Charlie-Silva et al. (2021), Gonçalves et al. (2022), Fernandes et al. (2022), Malafaia et al. (2022), Freitas et al. (2022), Freitas et al. (2023), Luz et al. (2023), and Tyrkalska et al. (2023). Such concerns are particularly associated with the spread of the virus in a wide range of aquatic ecosystems, especially in areas with a high incidence of COVID-19 and ineffective sewage treatment systems (Charlie-Silva & Malafaia, 2021; Malafaia et al., 2024). The reports by Yang et al. (2022), Pitol et al. (2023), Javanbakht et al. (2024), and Tandukar et al. (2024) on the persistence of viral RNA in water bodies justify studies on the possible ecotoxicological effects on aquatic organisms, even after the end of the COVID-19 pandemic and, consequently, the reduction of the risk of direct contamination.

On the other hand, less attention has been paid to the potential effects of aquatic contamination by SARS-CoV-2 on terrestrial organisms. The potential toxicity of the virus is not restricted to aquatic organisms but can also affect terrestrial organisms that interact with contaminated water environments, especially via ingestion of contaminated water, which can result in serious and potentially irreversible ecological consequences. However, studies available in the literature have mostly explored the direct and systemic effects of SARS-CoV-2 in murine models, focusing on how the infection affects multiple organs and systems (Leist et al., 2020; Winkler et al., 2020; Xia et al., 2021; Oh et al., 2022; Li et al., 2021; Luz et al., 2022; Lee et al., 2024) or the use of these models to test the efficacy of antiviral treatments, vaccines and/or to understand the effects of the infection in specific contexts, such as pregnancy and gastrointestinal dysfunctions (Bao et al., 2020; Dinnon et al., 2020; Lin et al., 2022; Zeng et al., 2022; Fontes-Dantas et al., 2023; Kim et al., 2023; Creisher et al., 2023; Li et al., 2024). These studies are crucial to understanding the pathological response and therapeutic interventions in controlled settings. However, they leave a gap in understanding the ecotoxicological effects of SARS-CoV-2 in terrestrial organisms exposed to contaminated aquatic environments. Limitations of the aforementioned studies that hinder the association with ecotoxicological effects include the predominance of models of direct exposure to SARS-CoV-2, mainly through intranasal or intraperitoneal inoculation, which need to adequately reflect environmentally relevant exposure routes. In addition, the focus on models used to evaluate therapies and vaccines and to test medical countermeasures distances the investigation from the ecological effects and indirect exposure of terrestrial organisms to aquatic contaminants.

Aiming to fill this knowledge gap partially, we recently evaluated the effects of ingestion of water containing SARS-CoV-2 lysis protein in terrestrial mammals (C57Bl/6J mice), demonstrating that oral exposure to this lysate induced behavioral disorders, oxidative stress, dopaminergic and cholinesterase imbalances in the brain, as well as hepatic, intestinal, and ovarian toxicity (Nunes et al., 2025). However, although the study by Nunes et al. (2025) provided valuable insights into systemic toxicity induced by viral components, it did not address the impacts on reproduction, mainly when exposure occurs before mating and gestation.

This critical phase can program long-term reproductive and behavioral functions. Given the importance of reproduction for the sustainability of populations (Chesson, 2018) and the possibility that pre-gestational exposure to SARS-CoV-2 may induce long-lasting effects, it is essential to investigate how this exposure may impact reproductive performance and maternal behavior. Assessments of this nature are necessary to detect and understand the impacts that prior exposure to the virus may have on the reproductive health of non-target mammals and remain relevant even after the end of the pandemic since the virus is still circulating and exposure to pathogens continues to be a frequent and unavoidable threat. Thus, in the present study, we evaluated whether the ingestion of water contaminated with SARS-CoV-2 in the pre-gestational period negatively affects reproduction in female mice based on the hypothesis that this exposure will result in adverse changes, such as changes in the length of the gestational period, reduction in the number of pups, changes in maternal behavior, and biochemical dysfunctions in critical tissues. Therefore, this study seeks to fill a crucial gap in understanding the indirect impacts of SARS-CoV-2 on non-target mammals, contributing to a broader understanding of the ecotoxicological risks associated with the pandemic and providing valuable information for environmental management and biodiversity preservation.

4.2 MATERIAL AND METHODS

4.2.1 SARS-COV-2 LYSATE PROTEIN

In this study, we used SARS-CoV-2 lysate protein (SARS.CoV2/SP02.2020.HIAE.Br, lineage B, GenBank: MT126808.1), provided by Dr. Edson Luiz Durigon (University of São Paulo, Brazil), isolated from the first confirmed case of COVID-19 in the country. The virus was collected from nasopharyngeal swabs of symptomatic patients who returned from a trip to Lombardy, Italy, in February 2020. After isolation, SARS-CoV-2 was cultured in Vero cells and analyzed by genomic sequencing and electron microscopy. The viral solution was prepared with a supernatant of Vero E6 cells in DMEM medium, with inactivated fetal bovine serum, and diluted in lysis buffer [see details presented in Araújo et al. (2020)]. The protein concentration was 0.9320 g/dL, measured with a commercial kit (kit BT1000900, lot 09S01A, Biotécnica Ind. e Com. LTDA, Varginha, Brazil), and the presence of the virus was confirmed by chromatographic immunoassay (Wondfo Biotech. Co. Ltd, USA).

4.2.2 ANIMALS AND EXPERIMENTAL DESIGN

As a non-target terrestrial mammal model for SARS-CoV-2 infection, we used nulliparous female C57Bl/6J mice (*Mus musculus*) raised at the animal facility of the Laboratory of Applied Toxicology for the Environment (Goiano Federal Institute, Urutaí Campus, Brazil). The animals were kept in ventilated boxes with controlled light (12h light/12h dark), temperature (24°C ± 2°C) and humidity (± 50%), with food and water ad libitum. The C57Bl/6J strain was chosen because it is inbred, reducing genetic variation in biological responses. In this study, we initially distributed 40 female mice (3.5-4 months) into two experimental groups (n=20 mice/group, n=10 animals/replicate) with equal ages and biomass. The "control" group consisted of female mice unexposed to SARS-CoV-2 lysate protein, and in the "SARS-CoV-2" group, the animals were exposed to SARS-CoV-2 lysate protein for 30 days via a drinking fountain diluted in drinking water (at 20 µg/L). The animals' boxes were cleaned every three days, and the water in the drinking fountains was renewed. According to Ferreira et al. (2023) and Luz et al. (2023), such concentration is considered predictive and environmentally relevant, simulating a pessimistic scenario of high discharge of domestic and hospital sewage, especially in regions with a high infection rate. To date, the studies mainly express the concentrations of SARS-CoV-2 in aquatic environments from equivalent viral RNA (genome copies/mL).

After the exposure period, all females underwent assessment of the estrous cycle phase using a combined approach: the visual method, which consists of inspecting the vulva to assess characteristics such as swelling, moisture, and coloration, and vaginal cytology, which involves collecting and microscopically analyzing vaginal cells to determine the cycle phase, as described by Ajayi & Akhigbe (2020). Females identified in the proestrus phase, a phase in which hormone levels increase, preparing the body for ovulation, and estrus, a period in which ovulation and maximum sexual receptivity occur (Zeng et al., 2023), were transferred to individual microisolators. This separation was intended to interrupt exposure to SARS-CoV-2 lysate protein and create ideal conditions for mating. Each female was paired with a male of reproductive age (6 to 10 months) who had not had previous contact with other females, thus ensuring the formation of a potentially reproductive pair and eliminating variables of prior behavior. The male was kept with the female for five days to ensure the female could mate during the proestrus and estrus phases. Although these phases last approximately 1.5 to 2 days, this period of cohabitation aimed to ensure that the male was present throughout the female's sexual receptivity period, maximizing the chances of mating. In addition, this duration allowed the synchronization of the male's reproductive behavior with the female's estrous cycle, optimizing the conditions for possible fertilization.

After mating, the presence of the vaginal plug, an indicator used to monitor mating success and the onset of pregnancy, was checked daily at noon. The visual assessment of the vaginal plug, as described by Şensoy & Öznurlu (2019), involved careful inspection of the perineal region of the females, where the presence of a whitish, mucous substance at the vaginal opening was taken as evidence of successful copulation and probable fertilization. The day on which the vaginal plug was detected was recorded as day 0 of gestation, also marking the time of removal of the male from the microisolator. The pups' birth days were monitored during the gestational period to calculate the gestation duration. At birth (always arriving at 7:00 p.m.), the total number of pups was counted, differentiating between live and stillborn pups, and the biomass of the live pups was measured. The Birth Biomass Index (BBI) was determined from Equation 1, which considers the total biomass of live pups, the total number of pups, and the length of gestation (in days). These factors were included to ensure that the index reflected individual pup biomass and the influence of litter size and gestational age, which can impact birth weight.

$$BBI = \frac{\text{Biomass (g)}}{(Total\ litter\ size) \text{ x (Gestational duration (days))}} \qquad \text{Eq. 1}$$

The day after birth, the females were subjected to a series of behavioral tests (as detailed in the subsequent item) and subsequently euthanized to perform biochemical analyses on different tissues, as described in item 2.4.

4.2.3 BEHAVIORAL TESTS

To comprehensively assess how exposure to SARS-CoV-2 lysate protein affected maternal behavior in females, both directly and indirectly, it was necessary to include both the open field and tail suspension tests to distinguish between general impacts on female behavior and more specific effects of exposure to the virus on maternal behavior. The order of the tests was strategically chosen to allow for an assessment of factors that could influence maternal behavior. The open field test was performed first to measure locomotor activity and reactivity to the environment, which could indicate baseline behavioral states in females. The tail suspension test was then performed to assess passive behaviors that could reflect a stress response. Although this test is known to be more stressful, it was a deliberate choice to perform it before the maternal behavior test. We chose this order so that the maternal behavior test, which is the main focus of the study, would be conducted after we had collected essential information about the behavioral and motivational state of the females. Performing the tail suspension test first allowed us to assess the potential impacts of passive stress, such as immobility and low motivation, which could affect maternal behavior. It is important to note that all tests described below were performed in a room specially equipped with soundproofing, temperature, and light control, as well as cameras connected to a computer that allowed monitoring of the animals in real time from outside the room.

4.2.3.1 Open Field Test

The open field test was performed as described in Guimarães et al. (2023), which consisted of introducing the animals individually into the center of a rectangular polypropylene arena with opaque white walls (dimensions: 16.5 cm high x 31 cm wide x 40.5 cm long) and filming them for 5 min. In the end, the Open Field Anxiety Index (OFAI) was determined, combining the time the animal spent in the central zone of the apparatus and the total number of crossings (virtually plotted on the computer screen (1 cm^2), reflecting the relationship between exploratory behavior and the propensity for anxiety-like behavior of the animals (Equation 2).

$$\text{OFAI} = \frac{\text{Time spent in the central zone (s)}}{\text{Total test duration (300 s)}} \div \frac{\text{Total number of quadrants crossed}}{\text{Maximum number of quadrants crossed}} \qquad \text{Eq. 1}$$

4.2.3.2 Tail Suspension Test

The tail suspension test, conducted immediately after the open field test, was performed using an apparatus similar to that described by Can et al. (2012). First, a plastic piece was placed around the mouse's tail to limit its ability to suspend itself; then, the tail was fixed to a metal rod with adhesive tape. The mouse was then suspended and filmed for 5 minutes, during which the immobility time was recorded, allowing the assessment of behaviors indicative of despair or low motivation.

4.2.3.3 Maternal Behavior Test

After the tail suspension test, the females were returned to the microisolators where they had given birth, along with their pups, which had been kept under artificial heating (provided by incandescent lamps with a yellow light spectrum) during the previous tests. The females and their pups remained in the test room for 10 minutes for acclimatization. Then, the mother was temporarily removed, and all the pups were removed (and taken to the vivarium), except three (which appeared healthy and had visible milk lines), which remained in the nest for 5 minutes under artificial heating. Subsequently, two of the pups were placed in opposite corners of the nest, and the third was positioned at an intermediate distance between them and the nest; then, the mother was reintroduced into the microisolator and filmed for 10 minutes. From the recordings, the following parameters were recorded: the initial distance between the mother and each pup immediately after their reintroduction into the apparatus, the distance of each pup from the nest, the time required for the female to approach and smell the pups for the first time (indicating initial recognition), the time until the mother picked up each pup in her mouth and transported it to the nest, and the total time the female remained on the nest, adopting an arched back posture indicative of nursing behavior after transporting all the pups to the nest. The distance parameters were analyzed using screenshots of the video recordings, followed by processing and measurement of the images using ImageJ software. The time parameters were quantified using PlusMZ® v1 software. Then, the initial exploration speed indexes per pup (Vexpl), composed of the ratio between the distance from the mother and the time to interact with the pups (Equation 3), and the speed of transport of the pups to the nest (Vtransp) were calculated. The latter was calculated as the ratio between the sum of the distances of each chick to the nest and the total time spent by the mother to carry each one of them (Equation 4).

$$\text{Vexpl} = \frac{\text{Initial distance (cm) between the mother and the pup}}{\text{Time (s) required for the first interaction}} \qquad \text{Eq. 3}$$

$$\text{Vtransp} = \frac{\sum \text{Dtransp}}{\sum \text{Ttransp}} \qquad \text{Eq. 4}$$

Where "∑Dtransp" is the sum of the distances of each pup to the nest and "∑Ttransp" is the total time the mother spent carrying each of them.

4.2.4. BIOCHEMICAL ASSESSMENTS

4.2.4.1 Sample Collection And Processing

Although females underwent mating and gestation without additional exposure to SARS-CoV-2 lysate protein, we assessed whether prior exposure resulted in lasting biochemical alterations that could have influenced both reproductive performance and maternal behavior. To this end, after behavioral testing, females were euthanized by cervical dislocation and underwent craniotomy procedures to collect the brain, olfactory bulb, and pituitary/hypothalamus, as well as laparotomy to excise fragments of the liver, mammary glands, left adrenal gland, and left ovary. For the mammary glands, all glandular tissue on the left side, including glands located in the thoracic and inguinal regions, was dissected and collected as a single unit. Dissection was performed following skin removal, with particular attention paid to preserving the continuity of the glands throughout the different regions of the body. All tissues were then immediately homogenized and macerated in phosphate-buffered saline (PBS, pH 7.2, kept at 4°C) using a cell disruptor and centrifuged at 13,000 rpm for 10 minutes at 4°C to separate the cellular components. The supernatants obtained were filtered through membranes with 0.45 μm pores and stored at -80°C until biochemical analyses. It is important to note that the entire procedure was performed under low light conditions to minimize interference in oxidative and antioxidant reactions, ensuring the integrity of the biomarkers to be evaluated.

4.2.4.2 Biochemical Biomarkers

4.2.4.2.1 Dopaminergic assessment

Dopamine levels in central nervous system tissues were assessed due to the central role of dopamine in regulating maternal behaviors, especially regarding motivation, reward, and adaptive response to pup care (Henschen et al., 2013; Xie et al., 2023; Li, 2023). For this, we used the colorimetric method based on the inhibition of TMB (3,3',5,5'-tetramethylbenzidine) oxidation by ceric ammonium nitrate (IV), adapted from the protocols of Wang et al. (2017) and Liang et al. (2021), with specific modifications to optimize experimental conditions. Briefly, 115 µL of ceric ammonium nitrate solution (14 mg/mL) was mixed with 50 µL of the supernatant of each sample. For dopamine controls, 50 µL of a dopamine solution (10 mM) was added. Then, 60 µL of TMB solution (0.01 M) was added, and the mixture was incubated at room temperature for 3 minutes. The absorbance of the samples was measured at 630 nm, and the dopamine concentration was determined from the equation of the straight line obtained by a standard curve prepared with dopamine concentrations ranging from 0 to 30 µM. The results were expressed as the percentage of inhibition of TMB oxidation, reflecting the dopamine levels in the analyzed tissues.

4.2.4.2.2 Cholinesterasic assessment

The activity of the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) is crucial for the regulation of acetylcholine levels in the central nervous system, an essential neurotransmitter for motor control, modulation of learning, memory, and behavior (Darvesh et al., 2003; Silman & Sussman, 2008). Therefore, to infer the potential cholinesterase or anticholinesterase effect resulting from ingesting water containing SARS-CoV-2 lysate protein, the activity of these enzymes was evaluated according to the protocol described by Ellman et al. (1961). The results were expressed both by the individual activity of the enzymes and by the AChE/BChE ratio to identify possible imbalances in cholinergic regulation that could impact neurological and behavioral functions.

4.2.4.2.3 Oxidative and nitrosative stress assessment

The levels of oxidative and nitrosative stress in the tissues of the central nervous system and other tissues were evaluated to identify possible relationships between these biochemical changes and maternal behavior. For this, reactive oxygen species (ROS) was quantified based on the protocol adapted from Freitas et al. (2023). The production of nitric oxide (NO) was evaluated through the indirect quantification of nitrite using the method proposed by Grisham et al. (1996), modified by Araújo et al. (2022), which is based on the Griess colorimetric reaction.

4.2.5. DATA ANALYSIS

Data normality and homoscedasticity were initially assessed using the Shapiro-Wilk and Levene tests, respectively. Comparisons between groups were performed using Student's t-test when the assumptions for using parametric models were met and using the Mann-Whitney test when these assumptions were not met. To assess possible correlations between dopamine levels in central nervous system (CNS) tissues and behavioral variables that showed differences between groups, data from both variables were pooled, log10-transformed, and normalized. Since the assumptions for parametric analyses were not met, we used Spearman's correlation coefficient (rs) with the permutation-based p-value. The significance level adopted for all analyses was 95%, and the data were processed and analyzed using GraphPad Prism 9 software (GraphPad Software, 2024) and Paleontological Statistics (PAST) (Hammer & Harper, 2001).

4.3 RESULTS

Initially, the analysis of reproductive parameters indicated that female mice did not present significant differences in gestational duration (Figure 16A), total litter size, number of live-born pups, and number of stillbirths (Figure 16C), and BBI also did not differ between the experimental groups (Figure 16B). On the other hand, although the tail suspension test did not reveal induction of passive behaviors in female mice by exposure to SARS-CoV-2 lysate protein in the pre-gestational period (Figure 17B), the anxiety index in these animals was significantly higher compared to the unexposed group. As shown in Figure 17A, the OFAI presented by the SARS-CoV-2 group was 70.9% higher than the control group. Furthermore, we observed that females in the SARS-CoV-2-exposed group interacted with the pups more quickly during initial exploration (Figure 17C), transported them more rapidly to the nest (Figure 17D), and exhibited nest attendance behavior for longer after pup transport (Figure 2D). The speed at which the animals were transported, in particular, was positively and significantly correlated with OBFAI (Spearman's ρ = 0.36; p = 0.0401).

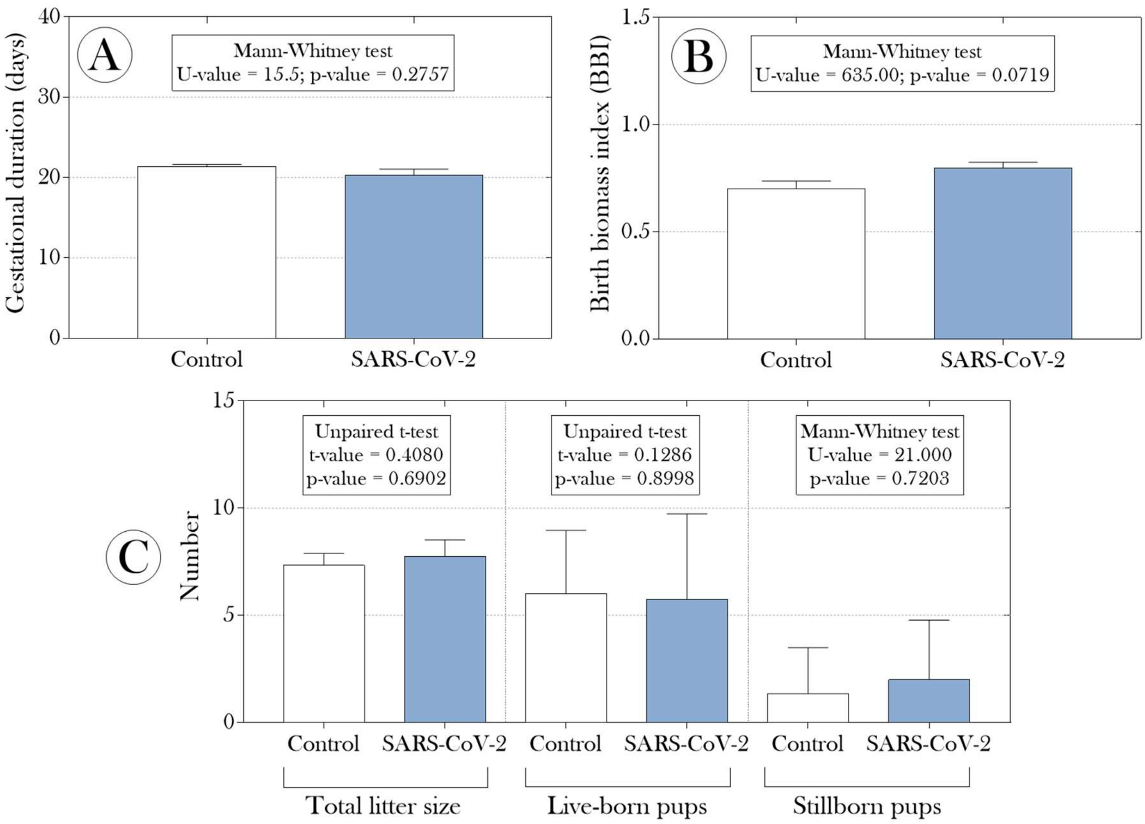

Figure 16. (A) Gestational duration, (B) Birth Biomass Index (BBI), and (C) total litter size, number of live-born and stillborn pups of female C57Bl/6J mice recently given birth unexposed (control group) or exposed to SARS-CoV-2 lysate protein, during the 30 days before mating and gestation period (SARS-CoV-2 group). Parametric data are presented as mean + standard deviation, while non-parametric data are presented as median and interquartile range.

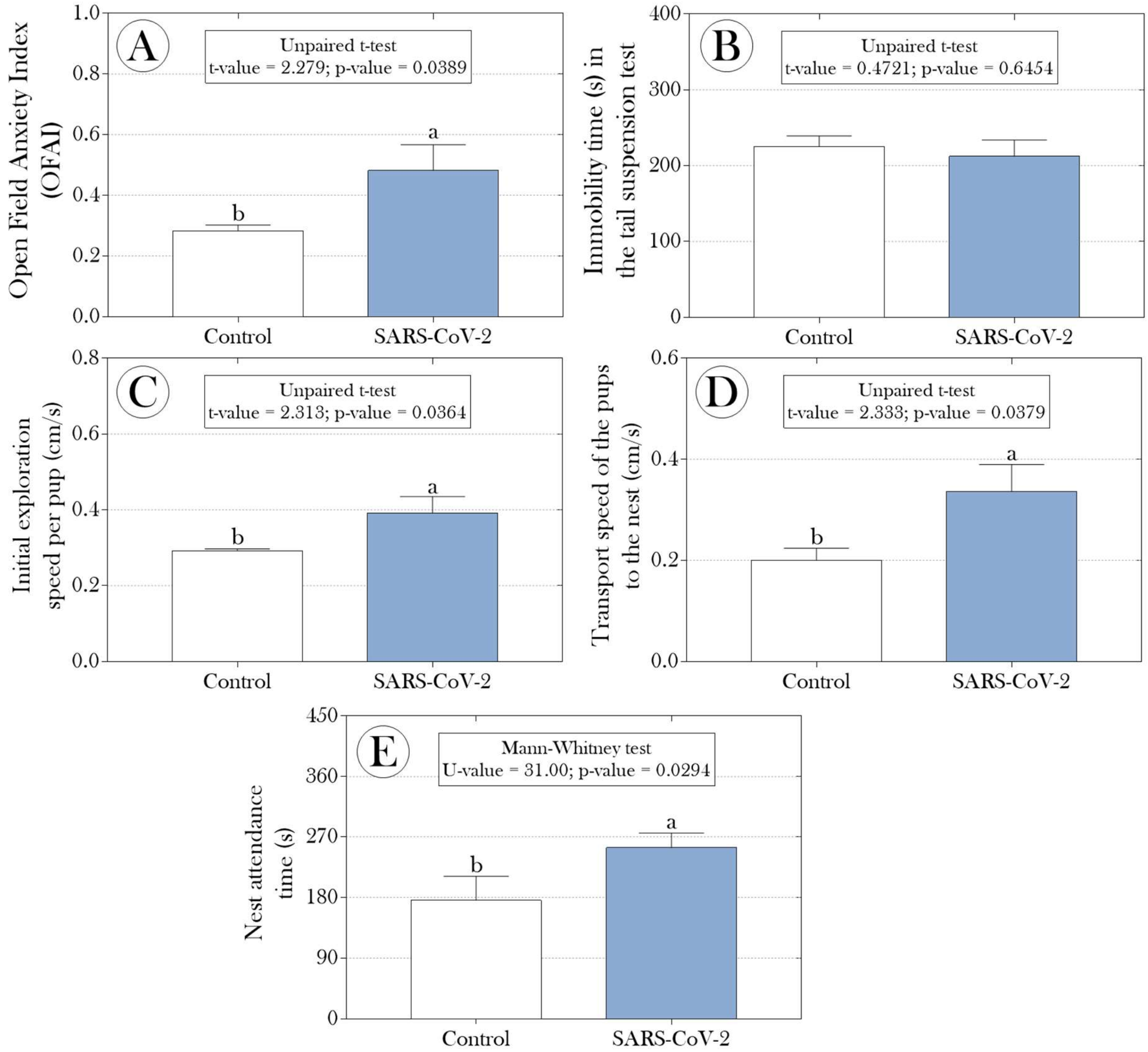

Figure 17. (A) Open field anxiety index (BBI), (B) immobility time (s) in the tail suspension test, (C) initial exploration speed per pup (cm/s), (D) transport speed of the pups to the nest (cm/s) and (E) nest attendance time (s) of newly-parous female C57Bl/6J mice unexposed (control group) or exposed to SARS-CoV-2 lysate protein, during 30 days before mating and gestation period (SARS-CoV-2 group). Parametric data are presented as mean + standard deviation, while non-parametric data are presented as median and interquartile range. Distinct lowercase letters indicate significant differences between the experimental groups.

Dopamine levels and cholinesterase activity were assessed in different CNS tissues to determine the relationship between these behavioral differences and possible biochemical alterations. As shown in Figure 18A, female mice from the SARS-CoV-2 group presented significantly higher dopamine levels in the olfactory bulb, brain, and region comprising the pituitary/hypothalamus than those observed in the control group. Dopamine levels in the pituitary/hypothalamus region, in particular, were positively and significantly correlated with OFAI (Spearman's $\rho = 0.58$; $p = 0.0268$), the speed at which females transported their offspring to the nest (Spearman's $\rho = 0.56$; $p = 0.0017$), and the display of nest attendance behavior

(Spearman's $\rho = 0.39$; $p = 0.0452$). Furthermore, we observed that both OFAI and the speed at which females interacted with pups during initial exploration increased proportionally to brain dopamine levels (Spearman's $\rho = 0.36$; $p = 0.0401$ and $\rho = 0.40$; $p = 0.0256$, respectively). Furthermore, we observed that ingesting water containing SARS-CoV-2 lysate protein altered the activity of AChE and BChE in the evaluated tissues. AChE showed increased activity in the olfactory bulb and reduced activity in the pituitary/hypothalamus region in the SARS-CoV-2 group (Figure 18C), while increased BChE activity was observed in both the olfactory bulb and the brain compared to the control group (Figure 18D). However, the ratio between the activity of these enzymes (AChE/BChE) did not differ between the experimental groups (Figure 18E). On the other hand, in the predictive analyses of oxidative and nitrosative stress, our data revealed organ/tissue-dependent alterations. While ROS production was significantly higher in the mammary glands and ovaries of females exposed to SARS-CoV-2 lysate protein (Figure 19A), nitrite levels were elevated only in the mammary glands, compared to the control group (Figure 19B).

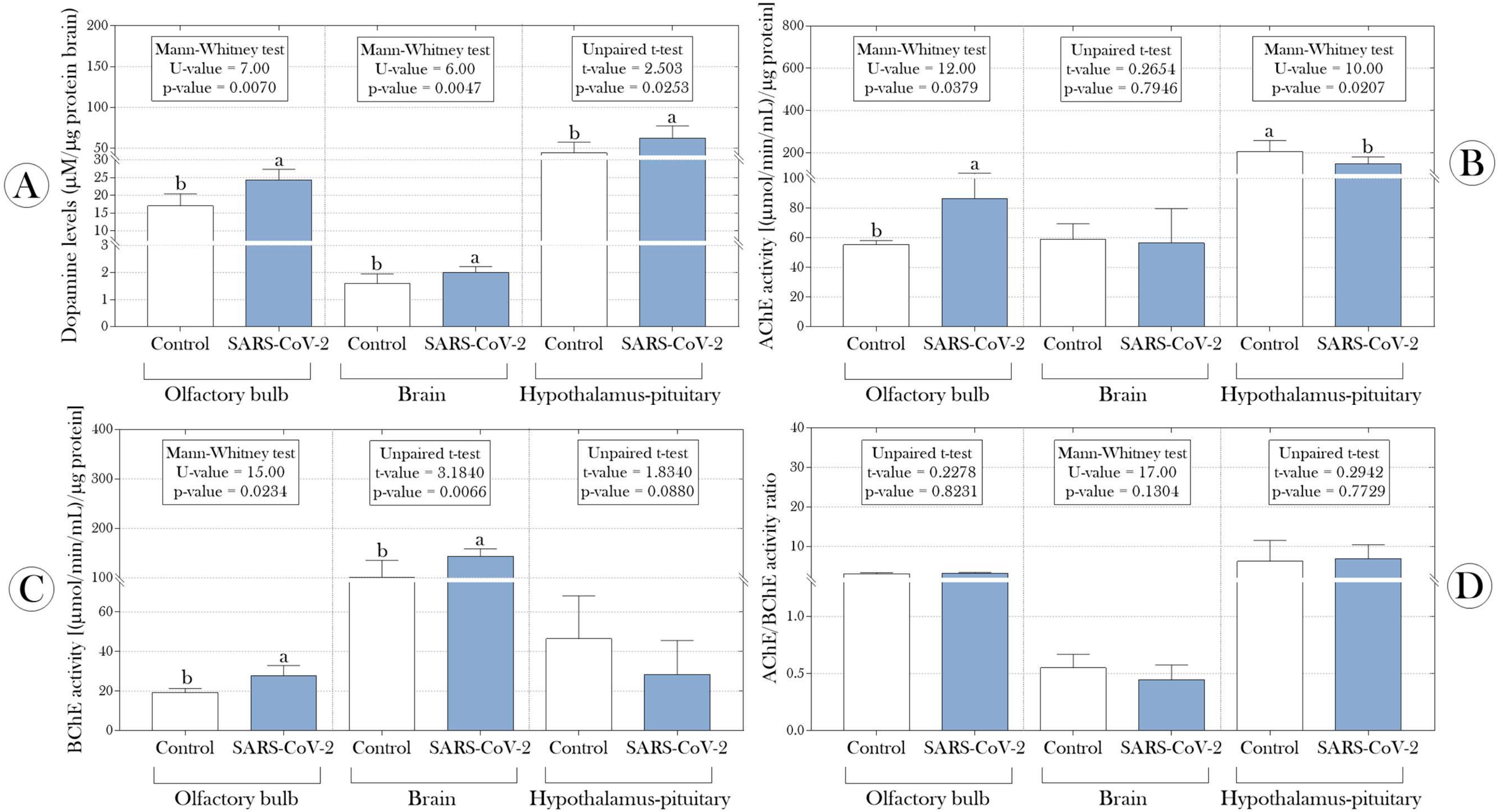

Figure 18. (A) Dopamine levels, (B) acetylcholinesterase (AChE) activity, (C) butyrylcholinesterase (BChE) activity, and (D) AChE/BChE activity ratio in the olfactory bulb, brain, and hypothalamus/pituitary (Hyp.-Pit.) region of female C57Bl/6J mice unexposed (control group) or exposed to SARS-CoV-2 lysate protein during the 30 days before mating and gestation period (SARS-CoV-2 group). Parametric data are presented as mean + standard deviation, while non-parametric data are presented as median and interquartile range. Lowercase letters indicate significant differences between experimental groups.

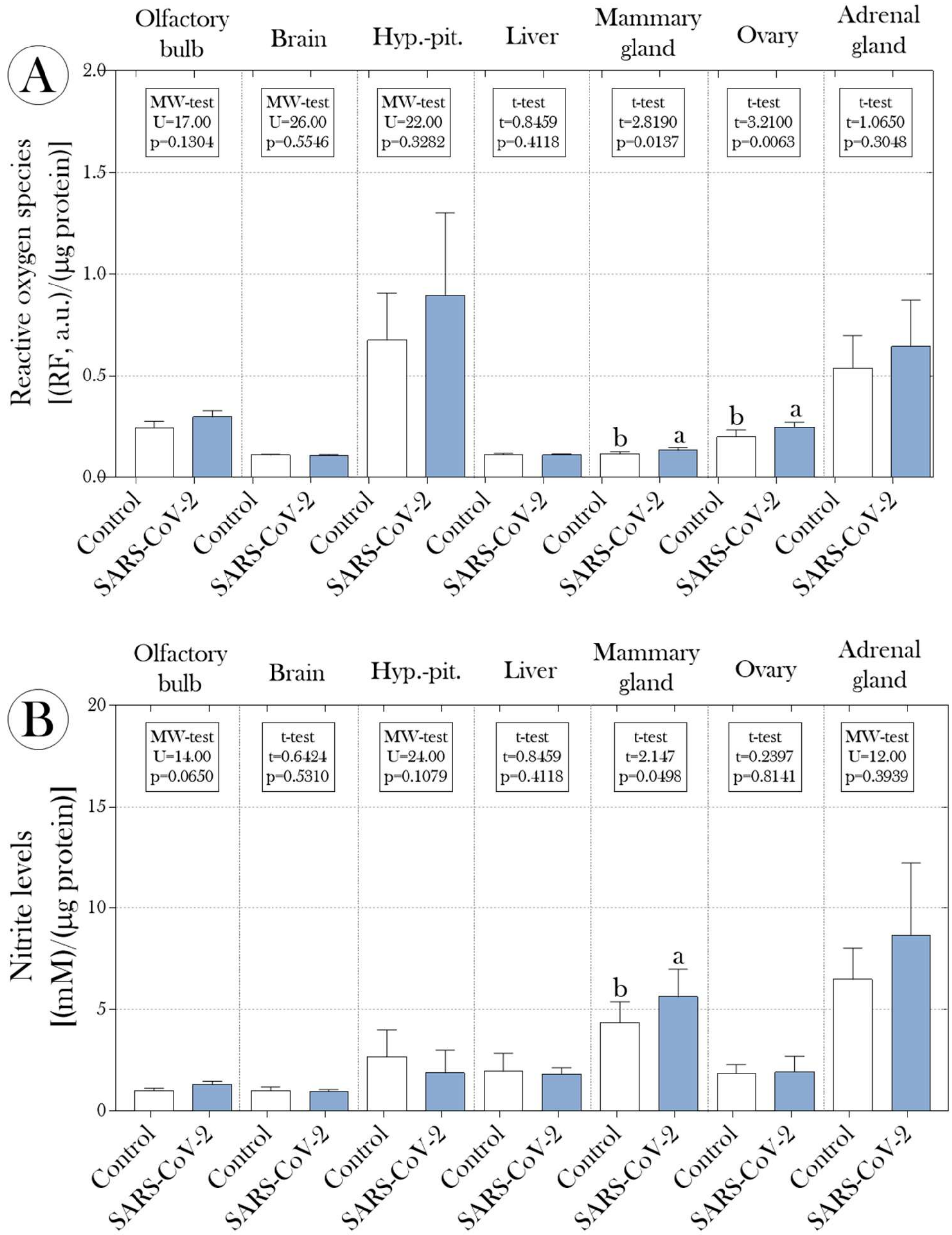

Figure 19. Production of reactive oxygen species (ROS) and nitrite in different tissues/organs [olfactory bulb, brain, hypothalamus/pituitary gland (Hyp.-Pit.), liver, mammary glands, ovary and adrenal gland] of female C57Bl/6J mice unexposed (control group) or exposed to SARS-CoV-2 lysate protein, during 30 days before mating and gestation period (SARS-CoV-2 group). Parametric data are presented as mean + standard deviation, while non-parametric data are presented as median and interquartile range. Lowercase letters indicate significant differences between experimental groups.

4.4 DISCUSSION

Contamination of terrestrial ecosystems by viral components, such as SARS-CoV-2 lysate protein, has become a relevant environmental concern, especially during and after the COVID-19 pandemic. Although some studies have addressed the effects of exposure to SARS-

CoV-2 or its specific peptides in aquatic organisms, the potential impacts on non-target terrestrial mammals within a broader ecological context still need to be explored. As discussed by Malafaia et al. (2024), the interaction of organisms with non-chemical toxic agents, such as human pathogens introduced into aquatic environments via domestic and hospital sewage, represents a significant exposure route. These pathogens, including bacteria, fungi, and viruses (such as SARS-CoV-2), can induce various ecotoxicological effects, such as behavioral and reproductive alterations. In this context, we investigated the effects of ingesting water contaminated with SARS-CoV-2 lysate protein in C57BL/6J female mice, emphasizing reproductive and behavioral implications. The main findings indicate that, although no significant changes in gestation length or litter size were observed, there were behavioral changes, including increased anxiety and changes in maternal behavior, reflecting interactions between elevated dopamine levels and altered cholinergic responses.

Initially, the results of our study demonstrate that pre-gestational exposure to SARS-CoV-2 lysate protein did not induce significant changes in the reproductive performance of C57BL/6J female mice, including gestation length, litter size, and pup biomass (Figure 16). Therefore, these findings suggest that there was no adverse impact on critical processes such as ovulation, sperm transport through the fallopian tubes to the fertilization site, fertilization itself, embryo implantation in the uterus, and maintenance of pregnancy. However, these results contrast with those reported by Nunes et al. (2025), who observed significant damage in the ovarian tissue of C57BL/6J female mice also exposed to SARS-CoV-2 lysate protein, including a reduction in the number of primary follicles, increased oxidative stress and inflammation, in addition to several histopathological alterations. The preservation of reproductive performance observed in our study suggests that physiological mechanisms have compensated for the adverse effects of exposure to SARS-CoV-2 lysate protein, highlighting the complexity of the responses and the need for further studies on reproductive function under conditions of viral stress.

On the other hand, the results of behavioral tests revealed significant changes that suggest neurobiological effects of pre-gestational exposure to SARS-CoV-2 lysate protein, even after cessation of exposure. The significant increase in the anxiety index observed in the open field test (Figure 17A) indicates greater sensitivity to stress in exposed females, possibly resulting from persistent neurochemical changes, mainly related to dopaminergic hyperactivity in the brain and hypothalamic-pituitary region (Figure 18A), areas crucial in the regulation of the stress response and anxiety-like behavior (Hinds & Sanchez, 2022; Csabafi et al., 2023). As reported by Yorgason et al. (2013) and Fernandes et al. (2021), increased dopaminergic signaling is strongly associated with the exacerbation of anxiety-like behavior in rodents since different dopaminergic systems in the brain, including the mesolimbic, mesocortical, and nigrostriatal systems, are involved in the modulation of anxiety. When dopamine levels are high, dopaminergic receptors have greater activation, especially D1 and D2, which can intensify reactivity to stressful situations, leading to an exaggerated state of alert and amplified anxiety-like behavior (Zarrindast & Khakpai, 2015). This dopaminergic hyperactivity may explain the increase in the anxiety index observed in females exposed to SARS-CoV-2 lysate protein. The faster initial interaction with the chicks, their transport to the nest, as well as the increased time of nest attendance (Figures 17C-E) may also be associated with elevated dopamine levels, known to influence maternal behavior and increase motivation for parental care (Henschen et al., 2013; Xie et al., 2023; Li, 2023). Although these results may initially suggest more acute and efficient maternal behavior, they may reflect a complex interaction between anxiety-like behavior and dopaminergic hyperactivity. This hyperactivity may have intensified maternal responsiveness, leading to a behavior that, although at first glance, seems more efficient, may reflect a state of exaggerated vigilance and a compensatory attempt to protect the offspring in an environment perceived as threatening due to previous exposure to SARS-CoV-2 lysate protein. Therefore, altered dopaminergic regulation plays a central role in modulating these behaviors, highlighting the complexity of neurochemical interactions that influence maternal behavior following exposure to SARS-CoV-2 lysate protein.

The changes in AChE and BChE activities in different tissues suggest a complex cholinergic modulation in the behavioral response of females after exposure to SARS-CoV-2 lysate protein. However, the relative balance between them remained unchanged between groups (Figure 18D). The increase in AChE and BChE activities in the olfactory bulb (Figures 18B-C) may have contributed to greater efficiency in the regulation of acetylcholine (ACh), resulting in a sensory sharpening that enhanced the responsiveness of females, leading to a faster initial interaction with pups (Figure 17C). On the other hand, the reduction in AChE in the hypothalamic-pituitary region (Figure 18B) may have led to an accumulation of ACh, altering the regulation of hormones and neurotransmitters crucial for emotional behavior and stress, which may be related to the increased anxiety observed in exposed females (Figure 17A). These enzymatic alterations suggest a long-lasting impact of viral exposure, indicating a possible adaptive reprogramming of the cholinergic system with long-lasting consequences for maternal behavior.

Our data also revealed increased ROS production in the mammary glands and ovaries of females exposed to SARS-CoV-2 lysate protein (Figure 19A), as well as an increase in nitrite levels (an indirect indicator of NO) in mammary tissue (Figure 19B), suggesting a persistent inflammatory response even after cessation of viral exposure. It is possible that the increased ROS and NO production was a consequence of a continuous activation of oxidative and nitrosative pathways triggered by a process of epigenetic reprogramming or changes in cellular signaling induced by exposure to the virus. Consequently, this reprogramming may have led to chronic low-grade inflammation that persisted throughout gestation and postpartum, affecting mammary and ovarian tissue. Although there are no studies similar to ours, Barenque et al. (2005), Ren et al. (2020), Gorini et al. (2021), Barbier et al. (2023), and Bezerra et al. (2023) corroborate this hypothesis by demonstrating that cellular signaling pathways associated with oxidative stress and inflammation can persist even after a long period of exposure to different pollutants/contaminants. This increased production of ROS in mammary and ovarian tissues may have influenced female behavior, especially through hormonal dysfunctions and changes in neuroendocrine signaling. As discussed by Ter-Horst et al. (2012) and Wieczorek et al. (2023), the ovaries are essential for the production of sex hormones, such as estrogen and progesterone, which not only regulate the reproductive cycle but also play a significant role in modulating behavior. Therefore, it is plausible that the oxidative stress observed in the ovaries, even if it did not affect reproductive performance, may have altered estrogen production. Small changes in estrogen levels may be enough to trigger behavioral imbalances, increasing the predisposition to anxiety (Kundakovic & Rocks, 2022). Furthermore, the nitrosative stress observed in the mammary glands and ovaries indicates an inflammatory condition that may have interfered with neuroendocrine signaling, releasing pro-inflammatory cytokines into the systemic circulation. These cytokines, capable of crossing the blood-brain barrier, can activate the HPA axis, leading to an increase in the release of cortisol (the main stress hormone) and exacerbating anxiety-like behavior, in addition to promoting an upward modulation of dopaminergic systems. This may explain the elevated dopamine levels observed in females of the SARS-CoV-2 group (Figure 18A) and the changes in maternal behavior (Figure 17), which may reflect a hyperactive and dysregulated response to stress.

Of course, it is essential to recognize that our study is not exhaustive, and the insights obtained should serve as a starting point for future studies that seek not only to overcome our limitations but also to explore in more detail the neurobiological mechanisms underlying the reported alterations. In our study, toxicity assessment was performed exclusively after the cessation of exposure, which did not allow us to investigate the potential effects of continued exposure during critical phases such as mating and gestation. Furthermore, the impacts of pre-gestational maternal exposure on pups were explored only based on biomass, without evaluating other parameters that could provide a more comprehensive understanding, such as the neurological and behavioral development of the pups. Although the concentration used in our study was environmentally relevant, future studies should consider applying different concentrations and including a broader range of toxicity biomarkers to obtain a more complete view of the potentially adverse effects. We also did not address individual variations in susceptibility to exposure, which could reveal differentiated responses and be better understood in studies involving different strains or species. To overcome these limitations, we suggest that future studies adopt more comprehensive approaches, such as conducting continuous exposures throughout the reproductive cycle, including mating, gestation, and lactation, to assess long-term effects at all critical stages. Neuroimaging techniques, such as functional magnetic resonance imaging (fMRI), may help determine changes in the neurological development of exposed pups. Additional behavioral tests, such as the radial maze test to evaluate working memory and spatial learning, the object recognition test to examine memory and familiarity, and the forced swim test to measure stress response and depression, may provide a more complete analysis of behavioral impacts. It is also recommended that genetically diverse mouse strains be used to investigate how genetic variation influences susceptibility to SARS-CoV-2 lysate protein exposure. Such approaches would significantly expand the understanding of the risks associated with ingesting water containing SARS-CoV-2 lysate protein.

In any case, our study showed that exposure of females to SARS-CoV-2 lysate protein resulted in behavioral and neurochemical changes that persisted after the end of exposure, with possible significant ecological implications. When extrapolating these results to wild populations of terrestrial mammals, it is likely that such behavioral changes could negatively impact population dynamics. The increase in the speed with which females initially interact with their offspring and transport them to the nest, although at first glance it may seem like an improvement in maternal behavior, can introduce considerable risks in natural environments. This acceleration in the execution of maternal behaviors, without due caution and exploration of the environment, can increase the vulnerability of both mother and offspring to predation. In addition, the increase in anxiety-like behavior in females, manifested by greater speed in actions, can compromise the safety of offspring, directly affecting survival rates and, ultimately, the resilience of populations over time. In a broader ecological context, these behavioral changes can also interfere with the ability of populations to compete for resources and reduce their ability to adapt to environmental changes. The combination of these factors can therefore decrease individual survival rates, but also compromise the viability of populations in the long term, leading to possible population declines. It is therefore crucial that future research investigates these dynamics, both in natural environments and under controlled laboratory conditions, to better understand the ecological risks associated with aquatic contamination by SARS-CoV-2 lysate protein and other non-chemical toxicants. Such studies are essential to predict and mitigate impacts on natural ecosystems, ensuring the preservation of biodiversity and the maintenance of ecological stability.

4.5 CONCLUSION

In conclusion, our study indicates that pre-gestational exposure to SARS-CoV-2 lysate protein in female C57Bl/6J mice did not result in significant alterations in reproductive performance, such as gestation length or litter size. These results, therefore, do not completely confirm the hypothesis that this exposure would cause adverse changes in these reproductive parameters. However, the hypothesis that exposure would result in behavioral and neurochemical alterations was confirmed. We observed a significant increase in anxiety-like behaviors and changes in maternal behavior, which were linked to elevations in dopamine levels and changes in cholinesterase activity in critical regions of the central nervous system. These neurochemical imbalances, combined with increased ROS and NO levels in mammary and ovarian tissues, indicate that exposure to SARS-CoV-2 lysate protein may affect both behavioral regulation and induce oxidative stress in reproductive organs, contributing to the observed alterations. Therefore, these findings reinforce the idea that, although there is no direct impact on reproduction, the ingestion of water containing SARS-CoV-2 lysate protein can generate long-lasting neurobehavioral and oxidative consequences, which, if extrapolated to wild populations, could negatively impact population dynamics and ecological resilience, especially in environments with continuous exposure to these non-chemical toxic agents.

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5 CONSIDERAÇÕES FINAIS

Este estudo amplia o conhecimento sobre os riscos ecotoxicológicos associados à dispersão do SARS-CoV-2 em ambientes aquáticos, fornece novos conhecimentos sobre os mecanismos de toxicidade da proteína do lisado do SARS-CoV-2 em um pequeno mamífero não-humano e, por conseguinte, expande a nossa compreensão da verdadeira extensão do impacto ecológico/ambiental da pandemia de COVID-19. A partir dos nossos dados, corroboramos que também há impactos negativos sobre organismos terrestres, o que pode ser potencializado em casos de exposição simultânea a outros poluentes, algo comum no meio ambiente.

Após a exposição oral ao lisado de SARS-CoV-2 (a 20 μg/L) por 30 dias, corroborou-se a suscetibilidade desses animais às proteínas virais, já que apresentaram alterações comportamentais, bioquímicas e alterações histológicas nos ovários, embora não tenham sido evidenciados efeitos hematológicos, mutagênicos e genotóxicos. Os distúrbios comportamentais observados foram a hipoatividade, comportamento semelhante à ansiedade e déficit de memória a curto prazo, que foram associados ao estresse oxidativo e ao desequilíbrio dopaminérgico e da colinesterase no cérebro dos animais. Em um ambiente natural, esses distúrbios representam mais riscos à predação e um menor forrageamento, já que estariam mais sedentários e estressados com a presença de possíveis predadores. Já a elevação dos níveis de bilirrubina e de desidrogenase láctica nestes animais sugere a ocorrência de alterações hepáticas, e o desequilíbrio redox, o estresse nitrosativo, a produção elevada de IFN-γ e o infiltrado inflamatório no duodeno, que, na natureza, poderiam estar relacionadas a uma desnutrição, impactando também a saúde dos adultos e a disponibilidade de nutrientes disponíveis aos filhotes em caso de uma gestação. Além disso, observou-se uma estrutura folicular perturbada, redução no número de folículos primários e a presença de vacúolos nas células da granulosa no ovário, impactos que podem repercutir negativamente na capacidade reprodutiva desses animais.

Contrariamente à nossa hipótese inicial, ao avaliarmos se haveria repercussões deletérias ao sucesso reprodutivo das fêmeas expostas durante sua puberdade, a exposição ao lisado viral realizada não repercutiu negativamente nos parâmetros reprodutivos avaliados (duração da gestação, tamanho da ninhada e biomassa da descendência ao nascimento). Em parte, essa ausência de resultados ocorreu pelo reduzido n amostral na segunda fase experimental, o que impediu a realização de análises estatísticas pareadas. Contudo, houve alterações neurocomportamentais significativas, com comportamento preditivo de ansiedade e alterações nos cuidados maternos, as quais estavam associadas a níveis elevados de dopamina e à alteração da atividade da colinesterase em regiões-chave do sistema nervoso central. Ainda, observou-se uma elevação de marcadores de estresse oxidativo e nitrosativo (ROS e NO) no ovário e nos tecidos mamários, sugerindo uma resposta inflamatória persistente. Portanto, os nossos dados evidenciaram que, mesmo após a cessação da exposição, as alterações neuroquímicas e comportamentais observadas podem ter efeitos duradouros. Em ambientes naturais, esses efeitos podem representar um risco significativo para a sobrevivência e estabilidade da população, principalmente através da modulação de vias neuroquímicas e respostas ao estresse em espécies não-alvo.

Dadas as limitações do nosso experimento, durante a segunda fase experimental, não pudemos avaliar os potenciais efeitos da exposição continuada durante fases críticas como o acasalamento e a gestação. Ainda, os impactos da exposição materna pré-gestacional nos filhotes foram explorados apenas com base na biomassa, sem avaliar outros parâmetros que poderiam fornecer uma compreensão mais abrangente, como o desenvolvimento neurológico e comportamental dos filhotes. Também não abordamos as variações individuais na suscetibilidade à exposição, que poderiam revelar respostas diferenciadas e ser melhor compreendidas em estudos que envolvam diferentes linhagens ou espécies. Para ultrapassar estas limitações, sugerimos que estudos futuros adotem abordagens mais abrangentes, como a realização de exposições contínuas ao longo do ciclo reprodutivo, incluindo o acasalamento, a gestação e a lactação, para avaliar os efeitos a longo prazo em todas as fases críticas. As técnicas de neuroimagem, como a ressonância magnética funcional (fMRI), podem ajudar a determinar as alterações no desenvolvimento neurológico das crias expostas. Testes comportamentais adicionais, como o teste do labirinto radial para avaliar a memória de trabalho e a aprendizagem espacial, o teste de reconhecimento de objetos para examinar a memória e a familiaridade, e o teste de natação forçada para medir a resposta ao stress e à depressão, podem fornecer uma análise mais completa dos impactos comportamentais. Também recomendamos a utilização de diferentes linhagens para investigar a forma como a variação genética influencia a suscetibilidade à exposição à proteína do lisado do SARS-CoV-2. Estas abordagens alargariam significativamente a compreensão dos riscos associados à ingestão de água contendo partículas virais do SARS-CoV-2.

Utilizando nossos resultados como subsídio, houve, ainda, o advento de novas questões para elucidação:

1. Há diferenças na ecotoxicidade entre cepas do SARS-CoV-2, seja do vírus propriamente dito ou do seu lisado viral?
2. Outras fontes secundárias de contaminação (ex. o lodo proveniente do tratamento de águas residuais) teriam um efeito similar ao observado em água contaminada?
3. Qual a atuação e importância de outros hormônios (ex. serotonina; hormônios reprodutivos) no comportamento materno? Seriam afetados pela exposição?
4. Testes comportamentais que avaliem comportamentos preditivos de depressão e de ansiedade em um contexto social propriamente dito teriam os mesmos resultados?
5. A associação de testes de comportamento materno (ex. avaliação da construção de ninho) teria resultados mais robustos?
6. Uma vez que houve alterações histopatológicas nos ovários das fêmeas expostas, também haveria alterações nos testículos dos machos?
7. A prole foi afetada pela exposição da mãe durante a sua juventude?

Uma vez que os camundongos foram utilizados como modelos experimentais de pequenos mamíferos terrestres, são considerados pragas quando em meio natural e dada a sua proximidade taxonômica com outras pragas urbanas, abre-se também a possibilidade de que outros animais terrestres que habitem ambientes expostos à contaminação por SARS-CoV-2 ou suas partículas virais também sofram efeitos adversos à sua saúde ou que também possam atuar como disseminadores ambientais do vírus, sendo, portanto, possíveis pivôs de surtos. Isto aliado à possibilidade de transmissão zoonótica reversa a gatos e furões, bem como os efeitos relativos à propagação de partículas virais a animais, abre a expectativa para consequências ambientais ainda desconhecidas e potencialmente perigosas à saúde pública e em uma abordagem integrada para prevenção à propagação de epidemias, ressaltando a importância da vigilância ambiental.

Nesse sentido, embora ainda seja incipiente e apenas tenha começado a se estabelecer em alguns países, como o Brasil, a perspectiva da Uma Só Saúde é uma promissora ferramenta auxiliar e integradora de saberes, fornecendo uma visão panorâmica sobre a realidade ambiental e a saúde. Pesquisas que visem elucidar lacunas de conhecimento e fomentar essa visão integral serão extremamente importantes para a criação de políticas públicas que sejam eficientes e justas socialmente, sem negligenciar a conservação ambiental.

APÊNDICE 1: SUPPLEMENTARY DATA. BEYOND THE VIRUS: ECOTOXICOLOGICAL AND REPRODUCTIVE IMPACTS OF SARS-CoV-2 LYSATE PROTEIN IN C57BL/6J FEMALE MICE

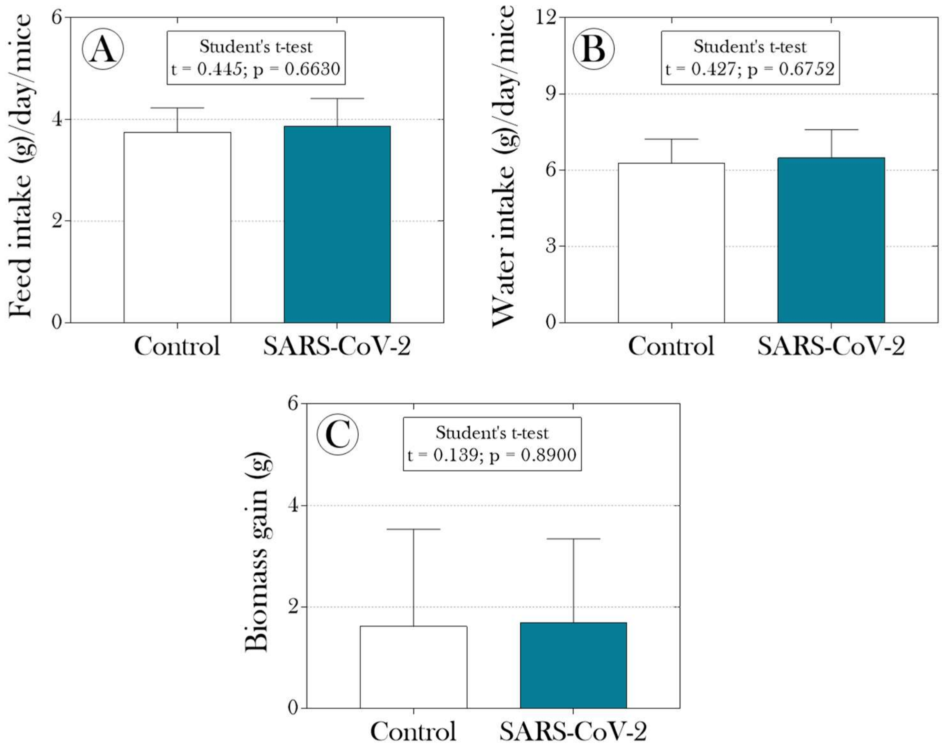

Figure S1. (A-B) Feed and water intake (respectively) and (C) biomass gain of C57Bl/6J female mice unexposed ("control" group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) ("SARS-CoV-2" group). Parametric data [compared by the Student's t-test (St test)] are presented by the mean + standard deviation. Statistical summaries are presented next to the graphs. Distinct lowercase letters indicate significant differences between experimental groups.

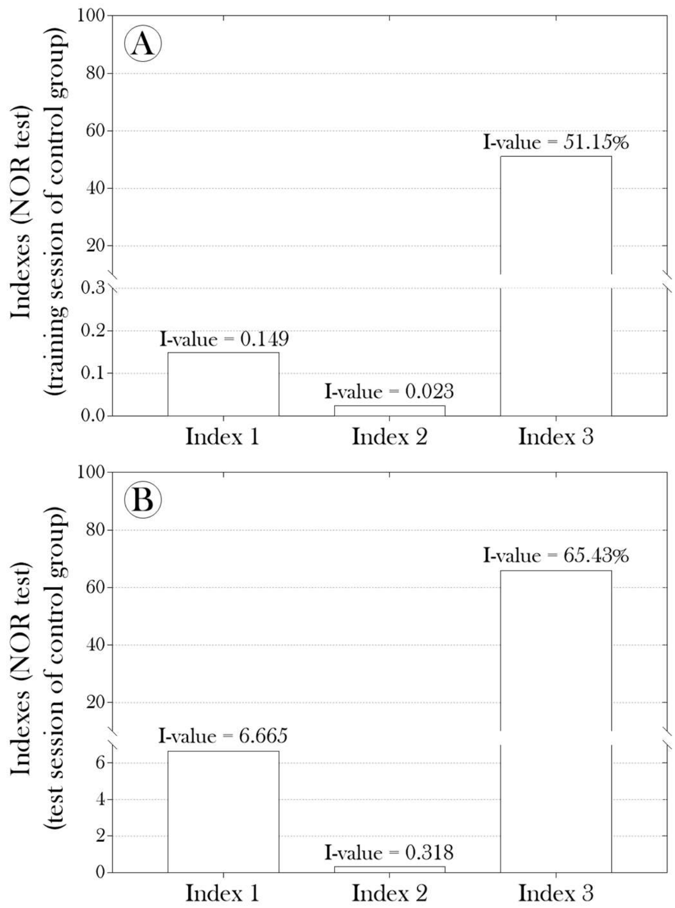

Figure S2. Object recognition index of C57Bl/6J female mice of the "control" group in the (A) "training" and (B) "test" sections, which demonstrate that the NOR test was validated.

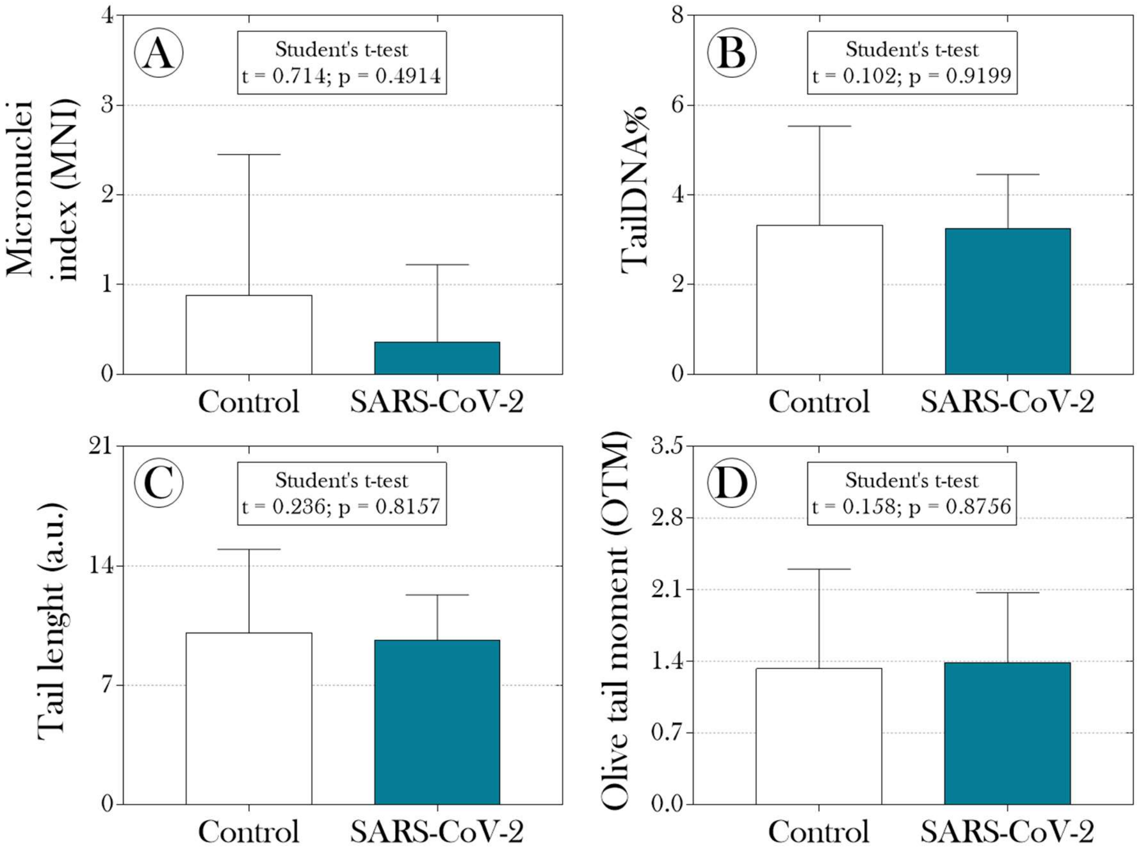

Figure S3. Biomarkers of mutagenicity [(A) Micronuclei index] and genotoxicity [(B) tail DNA %, (C) tail length, and (D) Olive tai moment] evaluated in C57Bl/6J female mice unexposed (“control” group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) (“SARS-CoV-2” group). Parametric data [compared by the Student's t-test (St test)] are presented by the mean + standard deviation. Statistical summaries are presented next to the graphs. Distinct lowercase letters indicate significant differences between experimental groups.

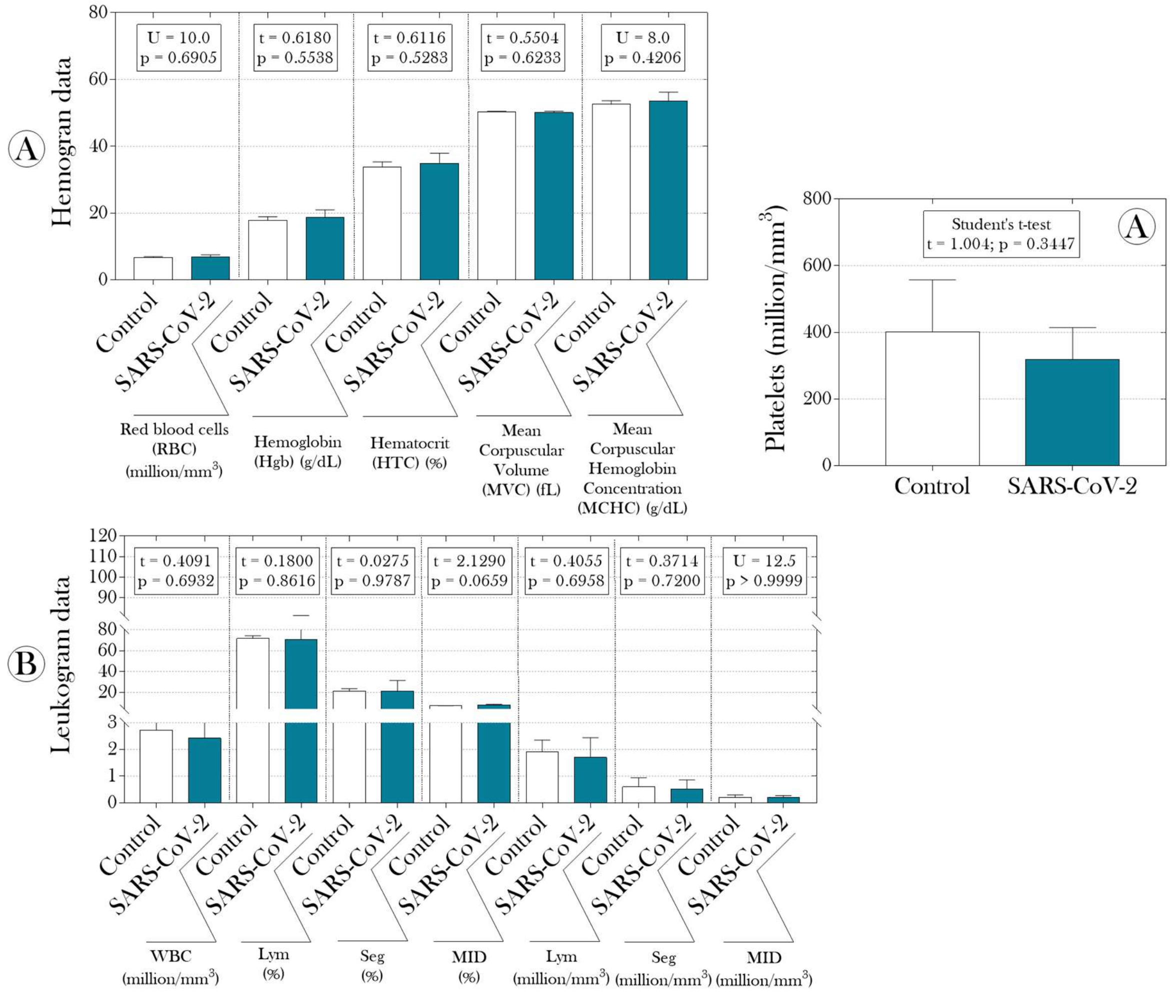

Figure S4. Hematological biomarkers [(A) hemogram and (B) leukogram data, and (C) platelets counts] evaluated in C57Bl/6J female mice unexposed ("control" group) or exposed to SARS-CoV-2 lysate protein (orally, to 20 µg/L) ("SARS-CoV-2" group). Parametric data [compared by the Student's t-test (St test)] are presented by the mean + standard deviation, whereas non-parametric data [compared by the Mann-Whitney test (MW test)] are presented by the median and interquartile range. Statistical summaries are presented next to the graphs. Distinct lowercase letters indicate significant differences between experimental groups.

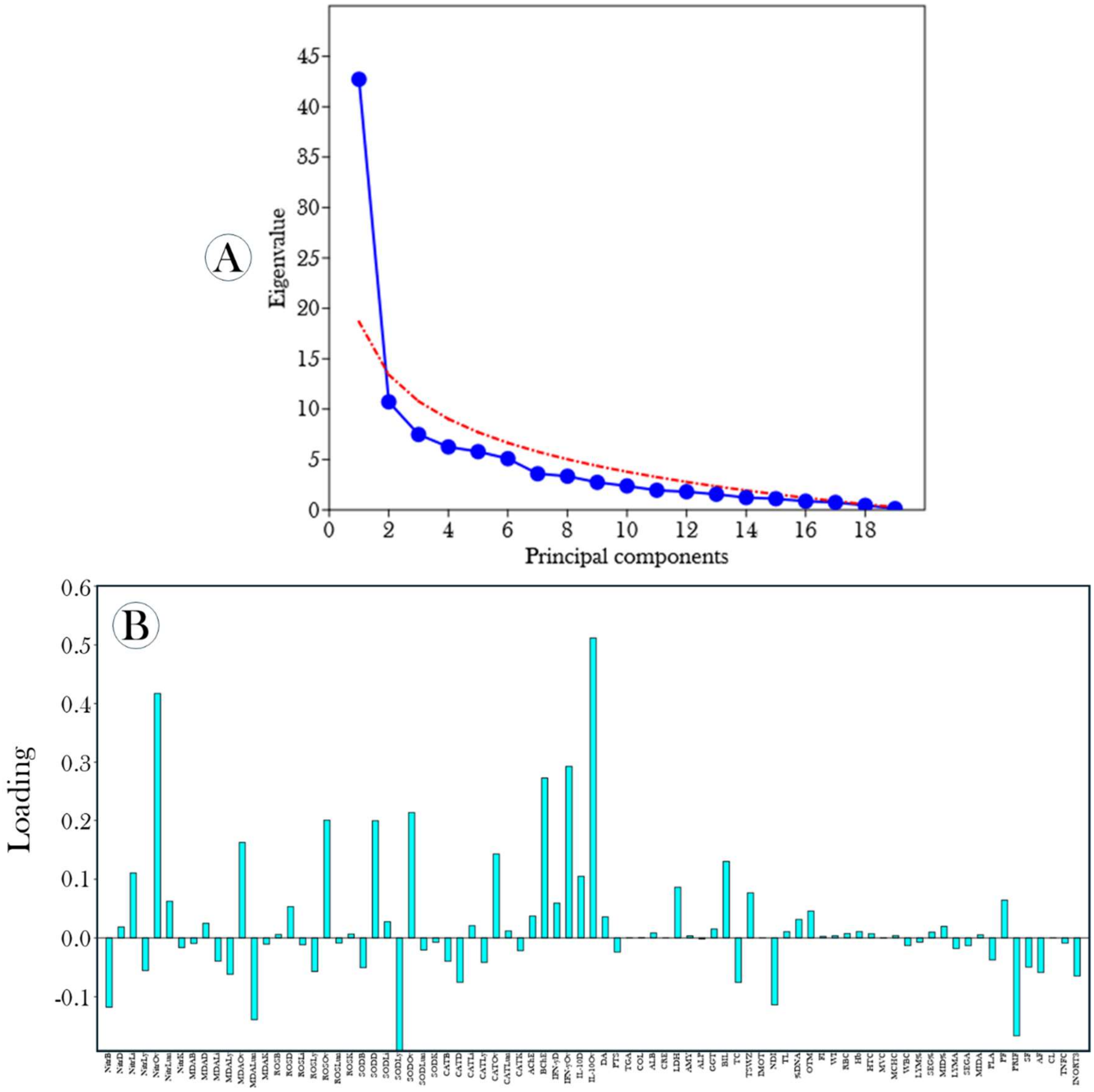

Figure S5. (A) Scree plot representing the eigenvalues and (B) bar plot of PC1 loadings of the variables accounted for by the principal components analysis (PCA). The meanings of the presented abbreviations are presented in Table S1.

Table S1. Biomarkers (and their respective abbreviations) used in principal component analysis (PCA) and cluster analysis.

ID	Abbreviations	Biomarkers
1	NitrB	Brain nitrite levels
2	NitrD	Duodenum nitrite levels
3	NitrLi	Liver nitrite levels
4	NitrLy	Mesenteric lymph nodes' nitrite levels
5	NitrOv	Ovarian nitrite levels

ID	Abbreviations	Biomarkers
6	NitrLun	Lung nitrite levels
7	NitrK	Kidney nitrite levels
8	MDAB	Brain malondialdehyde levels
9	MDAD	Duodenum malondialdehyde levels
10	MDALi	Liver malondialdehyde levels
11	MDALy	Mesenteric lymph nodes' malondialdehyde levels
12	MDAOv	Ovarian malondialdehyde levels
13	MDALun	Lung malondialdehyde levels
14	MDAK	Kidney malondialdehyde levels
15	ROSB	Brain reactive oxygen species levels
16	ROSD	Duodenum reactive oxygen species levels
17	ROSLi	Liver reactive oxygen species levels
18	ROSLy	Mesenteric lymph nodes' reactive oxygen species levels
19	ROSOv	Ovarian reactive oxygen species levels
20	ROSLun	Lung reactive oxygen species levels
21	ROSK	Kidney reactive oxygen species levels
22	SODB	Superoxide dismutase activity in the brain
23	SODD	Superoxide dismutase activity in the duodenum
24	SODLi	Superoxide dismutase activity in the liver
25	SODLy	Superoxide dismutase activity in the mesenteric lymph nodes
26	SODOv	Superoxide dismutase activity in the ovary
27	SODLun	Superoxide dismutase activity in the lung
28	SODK	Superoxide dismutase activity in the kidney
29	CATB	Catalase activity in the brain
30	CATD	Catalase activity in the duodenum
31	CATLi	Catalase activity in the liver
32	CATLy	Catalase activity in the mesenteric lymph nodes
33	CATOv	Catalase activity in the ovary
34	CATLun	Catalase activity in the lung

ID	Abbreviations	Biomarkers
35	CATK	Catalase activity in the kidney
36	AChE	Acetylcholinesterase activity in brain
37	BChE	Butyrylcholinesterase activity in the brain
38	IFN-yD	Duodenum interferon-gamma levels
39	IFN-yOv	Ovarian interferon-gamma levels
40	IL-10D	Duodenum interleukin-10 levels
41	IL-10Ov	Ovarian interleukin-10 levels
42	DA	Brain dopamine levels
43	TPS	Serum total protein levels
44	TGA	Serum triglycerides levels
45	COL	Serum total cholesterol levels
46	ALB	Serum albumin levels
47	CRE	Serum creatinine levels
48	LDH	Serum lactate dehydrogenase activity
49	AMY	Serum alpha-amylase activity
50	ALP	Serum alkaline phosphatase activity
51	GGT	Serum gamma-glutamyl transferase activity
52	BIL	Serum bilirubin levels
53	TC	Total crossings (open field test)
54	TSWZ	The ratio between time spent at the wall zone and total crossings (open field test)
55	IMOT	Immobility time (tail-suspension test)
56	TL	Tail length (comet assay)
57	%DNA	Tail DNA% (comet assay)
58	OTM	Olive tail moment (comet assay)
59	FI	Feed intake
60	WI	Water intake
61	RBC	Red blood cells
62	Hb	Hemoglobin

ID	Abbreviations	Biomarkers
63	HTC	Hematocrit
64	MVC	Mean corpuscular volume
65	MCHC	Mean corpuscular hemoglobin concentration
66	WBC	White blood cell
67	LYM%	Lymphocyte count (relative)
68	SEG%	Segmented neutrophils count (relative)
69	MID%	Monocytes (relative)
70	LYMA	Lymphocyte count (absolute)
71	SEGA	Segmented neutrophils count (absolute)
72	MIDA	Monocytes (absolute)
73	PLA	Platelets
74	PF	Primordial follicles
75	PRIF	Primary follicles
76	SF	Secondary follicles
77	AF	Antral follicles
78	CL	Corpus luteo
79	TNFC	Total number of ovarian follicles or corpus luteum
80	INORT3	Index 3 (novel object recognition test)