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Reč urednika / Word from the editor

Društvo za ishranu Srbije je jedna od najstarijih zajednica naučnih radnika i stručnjaka u Srbiji koja ima za cilj da bude nacionalni servis stručne i šire javnosti. Od svog osnivanja 1956. godine objedinjuje istraživače iz oblasti agronomije, biohemije, hemije, farmacije, humane i veterinarske medicine, dijetetike i drugih srodnih naučnih disciplina koji brinu o proizvodnji i bezbednosti hrane i zdravlju nacije, posebno dece.

Hrana i ishrana je pregledni časopis Društva za ishranu Srbije koji od 1960. godine prati osnovne postulate Društva u cilju poboljšanja i unapređenja javnog zdravlja. Časopis objavljuje originalne istraživačke i pregledne članke, teme za diskusiju, vesti i informacije, kao i ekspertske izveštaje o svim aspektima hrane i ishrane.

Časopis je mesto koje obezbeđuje da se glas naučne zajednice čuje i blagovremeno prenose informacije o hrani i ishrani stanovništvu, mesto za sve napredne ideje i kritičko razmišljanje.

Osnovni cilj časopisa **Hrana i ishrana** je integracija znanja fundamentalnih istraživanja nauke o hrani i ishrani u zdravlju i bolesti. Paralelno sa istraživanjem molekularnih mehanizama delovanja hrane, časopis je usmeren na praćenje zakonodavstva i etičkih standarda, što je danas veliki izazov u svetu kada se radi o hrani.

Uredнički tim očekuje interaktivnu saradnju i pomoć svih naučnika kojima je hrana i ishrana više od značenja tih reči, etička kategorija koja ima za cilj poboljšanje zdravlja nacije.

Bato Korać
Glavni i odgovorni urednik

Serbian Nutrition Society is one of the oldest communities of scientists in Serbia, which aims to be a national service and a service to all people. Since its establishment in 1956, it unites researchers, doctors, educators, and followers who care about the health of the nation, especially children.

The **Food and Nutrition** is a peer-reviewed journal of the Serbian Nutrition Society which since 1960 follows the basic postulates of the Society, with the aim to improve and promote public health. The Journal publishes original research and review articles, topics for discussion, news and information, and expert reports on all aspects of food and nutrition.

The Journal is a place that ensures that the voice of the scientific community is heard and timely transmits information related to food and nutrition to the population, the place for all the advanced ideas and critical thinking.

The primary aim of the **Food and Nutrition** journal is to integrate the knowledge of fundamental research of food science and nutrition in health and diseases. In parallel with the research of molecular mechanisms of action of food, the scope of the Journal is to monitor legislatures and ethical standards, which today is a great challenge in the world when food is concerned.

The editorial board expects interactive cooperation and assistance from all scientists to whom food and nutrition is more than the meaning of those words, an ethical category aimed at improving the health of the nation.

Bato Korać
Editor in Chief

Ukoliko želite da naručite određeni broj časopisa, možete se obratiti na sledeći mail: ljilja.t.p@gmail.com.

Ukliko želite da se učlanite u Društo za ishranu Srbije, neophodno je da popunite zahtev koji se nalazi na internet stranici Društva za ishranu Srbije www.hrana-ishrana.org i uplatite godišnju članarinu u iznosu od 1000 dinara na sledeći žiro račun 355-1032408-17.

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REVIEW PAPER

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COVID-19 as milestone for the use of new vaccine types

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Abstract

COVID-19 is an infectious disease caused by SARS-CoV-2 virus which belongs to the Coronaviridae group. The symptoms of the disease are primarily present in the form of respiratory syndrome similar to the symptoms of other respiratory viruses such as influenza virus but also the very common SARS-CoV-1 and MERS-CoV viruses known from the recent past. However, based on short clinical experience, it was found that apart from lungs COVID-19 affects all organ systems with unclear pathogenesis. Respiratory failure followed by systemic hypoxia and coagulation disorders is hallmarks of severe pathology. In addition, this virus belongs to the group of RNA viruses that share common characteristics with the HIV virus, which makes it very challenging for the therapy of modern medicine. There is currently controversy around the world about how to combat this epidemic. In addition to hygienic measures and measures of safe distance and isolation, there is an intense controversy about prophylactic vaccines. In this review article, the insight into all aspects of vaccination and methods for their design based on technological tools possessed by modern science with special reference to genetic (DNA, RNA) vaccines is provided, and the question arises of whether there is a justification of novel technology urgent application in the event of a COVID-19 pandemic.

Key words: *COVID-19; Vaccines; Live attenuated; VLP; Vector; Protein; DNA; RNA.*

INTRODUCTION

COVID-19 (SARS-CoV-2) infection broke out at the end of the 2019 year in Wuhan, China, and spread all over the world by 11 March of 2020 year when the global epidemic state is declared by the World Health Organization (WHO). According to the WHO statistics, this is one of the 20 infectious diseases which cause epidemic state in the past decade [1]. Many of them are followed by respiratory failures such as H1N1 [2] and MERS [3] so-called the novel coronavirus. In that manner, over the past years, there are a lot of challenges in front of health and science communities concerning the treatment of coronavirus-associated diseases (SARS and MERS) [4] to which is now added the COVID-19 [5]. In that context, one of the main issues which originate earlier than COVID-19 epidemics is the vaccine development for such contagious diseases. Speaking in general, the antiviral vaccines are divided into the next following types: inactive and/or live-attenuated viruses, virus-like particle (VLP), viral vectors, protein-based, DNA-based, and mRNA-based vaccines [6]. All the type of vaccines will be elaborated in detail in this review article.

LIVE ATTENUATED VACCINES

Inactive virus vaccines are made from the whole virus particles inactivated by formaldehyde or gamma irradiation, while the live-attenuated vaccines are developed in recombination with live attenuated viruses as a mutant [7]. In inactive virus/bacteria vaccines the virus or bacteria is functional but has been weakened, so it can replicate in the body several times and generate an immune response without causing the development of the disease. The example for such type of vaccines are chickenpox, measles, mumps and rubella, rotavirus, and shingles vaccine viruses, as well as the BCG vaccine which contains live weakened tuberculosis bacteria. If a live attenuated vaccine does cause disease, e.g., chickenpox disease, it is usually mild than

a disease caught by another person in the community [8]. But if administered to a person with an impaired immune system response, e.g., leukemia or HIV infection, or in those who are taking immunosuppressive medications, may cause severe disease as a result of uncontrolled replication and growth [9]. What is certain, attenuated viruses/bacteria in a form of vaccines when injected into the body induce the immune response, after which the virus particles are eliminated by innate immune mechanism and expelled from the body via kidney or liver, without causing the disease development. Both of these vaccines are in the pre-clinical phase of study for COVID-19 and need for several years to set up clinical use [10]. According to the WHO draft landscape of COVID-19 candidate vaccines, data collected up to the 12 November of 2020, there are 164 candidate vaccines in pre-clinical evaluation, and 48 candidate vaccines in clinical evaluation (**Table 1**). Only 3 study (Acıbadem Labmed Health Services, Serum Institute, and Griffith University all situated in India) deals with the live attenuated virus vaccines and just 4 of all study (one in Vietnam, one in Thailand, and two in Brazil) test a whole virus structure, and each of these trials are in the pre-clinical phase of evaluation (**Figure 1**). This way of developing vaccines has certain advantages such as good preservation of virus particle structure, rapid development, excellence in neutralizing antibody (Ab) induction, formulation with various types of adjuvant, and the most important is excellence in the induction of T and B cells responses [7]. It is known that inactive or live-attenuated vaccines are the most common way of immunization used in the past with great success. Particularly, this offers the best possible simulation to the natural way of pathogen introduction, minimizing the most of the eventual consequences of overall homeostasis disruption in interplay between host and pathogen. Apart from this, the priority in COVID-19 pandemic is given to new technology in vaccines design with only few trials for "old fashion" vaccines. To understand the inferior position of standard approach in the vaccine market, detail evaluation of main features, advantages and risks need to be reviewed.

VIRUS-LIKE PARTICLE VACCINES

Virus-like particles (VLPs) represent structures consisted of several proteins but without genetic material, by which they retain the organization of native viruses [11]. They are formed by the self-assembly of viral capsid proteins, and besides lacking viral genome retain the ability to enter the cells. VLPs are even convenient for fusion with other heterologous antigen epitopes, which makes them good as a platform for multimeric vaccine design [12]. So far, several types of vaccines based on VLP technology are known to be commercialized in the world, such as vaccines for hepatitis B virus as well as human papillomavirus [13]. It is known that different virus capsid protein might be able to efficiently assemble into various cells such as human embryonic kidney cell culture (HEK293), yeast, baculovirus, lentivirus, some bacteria such as *E. coli, Spodoptera frugiperda (sf9)* cells etc [11]. But on the other hand, despite the structure of virus capsid proteins are well preserved when synthesized in all those systems, the main disadvantage is that immunogenic VLPs require the optimum assembly conditions [7]. Furthermore, VLP vaccines designed in a form of multimeric antigen display in various cell cultures are different size ranging from 22 up to 150 nm [14]. Despite good preservation of virus particle structure in those systems, the variety of VLP particles in size is basically a problem when it comes to their immunogenicity. This might be a problem with COVID-19 VLP vaccines as well. Namely, one of the study deals with the VLP vaccine for COVID-19 produced in baculovirus, which is consisted of receptor-binding domain (RDB) with a full-length spike (S) or S1, co-expressing with M and E subunit as a part of virus structure [15]. In parallel, there are 16 studies in pre-clinical phase, and 2 studies in clinical phase according to WHO statistics (**Figure 1**, **Table 1**) dealing with COVID-19 VLPs made from different capsid protein such as envelope virus-like particles (eVLPs), S protein, RDB domain as well as unknown COVID-19 structures (Doherty Institute) (**Table 1**). All those VPL vaccines are produced by various cell cultures, starting from using plant derived VLP (Medicago Inc., Shiraz University), across VLP produced in baculovirus Expression Vector System BEVS (Tampere University), the form of lentivirus and baculovirus vehicles together, than VLP gained from cell cultures associated with hepatitis B virus antigen (HBsAg) (SpyBiotech/Serum Institute of India), or integrated in HIV VLPs (IrsiCaixa AIDS Research/IRTA-CReSA/Barcelona Supercomputing Centre/Grifols) (**Table 1**). In order to prime the immune response, some of them are combined with different adjuvants (Mahidol University/ The Government Pharmaceutical Organization (GPO)/Siriraj Hospital) or navigated to antigen presenting cells using particle-based Dendritic Cell (DC) targeting approach (University of Manitoba). The question is would all those COVID-19 VLPs vaccine be equally effective? All these VLP based vaccines are listed in **Table 1** with the information about the researchers groups and/or organizations involved in their production (data obtained from official website of WHO).

RECOMBINANT VIRAL VECTOR VACCINES

Recombinant viral vector vaccines represent corresponding viral component as a carrier in a form of retrovirus, lentivirus, vaccinia virus, adenovirus, ade-

no-associated virus, cytomegalovirus or sendai virus, transfected by specific antigens (transgene) from a virus of interest. This technology based on vector vaccines are characterized by certain advantages such as high efficiency of gene transduction, specific delivery of genes of interest to the target cells, and the induction of robust specific immune response. On the other hand, depending on the vector used for vaccine design, there are numerous pronounced disadvantages such as generation of replication-competent virus (retrovirus, lentivirus), tumorigenesis risk (retrovirus, lentivirus), limited infection of dividing cells only (retrovirus), pre-existing immunity (vaccinia virus, adenovirus, cytomegalovirus), low titre of antibody production (adeno-associated viruses), as well as the risk of pathogenesis in specific individuals (cytomegalovirus) [16]. Furthermore, by the process being called transgenic transfection the recombinant virus particles might also be detrimental in the context of deletion or inactivation of the transgene of interest itself which may occur either during the vaccine production process or during the immunization procedure [17]. The bright side of viral vaccines is that they do not require adjuvants as it is in case with protein vaccines, but there is concern about the theoretical reduction of desirable immunity and the possibility of carrier-induced infection. Studies evaluating the recombinant adenovirus type-5 (Ad-5) as a vector for HIV-1 vaccine reported increased risk for HIV-1 acquisition among vaccinated men [18-20]. Base on this results Zhu *et al*. express the concern about the usage of Ad-5 as a vector for COVID-19 phase 1 vaccine study [21]. Despite this, there are 5 studies for COVID-19 vaccines based on Adenovirus type-5 all listed in the **table 1**. Furthermore, among all vaccines for COVID-19, viral vectors vaccines occupy a central role with 37 studies which are in pre-clinical phase, and 13 already involved in clinical phase of testing (data obtained from WHO website).

PROTEIN-BASED VACCINES

Protein vaccines are composed of purified or recombinant proteinaceous antigens from a certain pathogen. The first protein-based vaccines originate from the plasma harvested antigens. In that manner, the first hepatitis B protein based vaccine is developed by the purification of viral surface 22-nm lipoprotein particle (HBsAg) from plasma collection of infected individuals [22]. However, limited source of HBsAg in plasma, despite the great tolerability and efficiencies of vaccine, demand more convenient recombinant technology in vaccine design.

The greatest advantage of protein-based vaccines is that they are safe in the context of development of infection, since they are consisted of targeted antigen with epitope responsible for triggering a specific immune response. On the other side, week immune response they are providing needs appropriate adjuvants in order to prime innate immune response [23]. Some of these stabilizers such as aluminium (Al) are used in a form of AlPO4, and this is accepted adjuvant in vaccination practice in many vaccines today starting from 1930 [24]. Usage of Al is supported by the fact that Al enhanced humoral responses priming to MHC class I molecules [25]. However, there are studies pointed out Al side effects indicating relation between Al toxicity and development of neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS), Alzheimer's disease (AD), dementia, Gulf war syndrome and Parkinsonism [26]. Tremendous escalation in development of autism spectrum disorder (ASD) in last decades is brought into tight connection with Al support in vaccines and is a question of serious debate in science today [24, 27-30]. Despite these data available in public, Al is as used as a stabilizer (KM Biologics, Shifa Pharmed) in COVID-19 vaccines [10] and some of them are in phase 3 of clinical trial NCT04560881 sponsored by Laboratorio Elea Phoenix S.A. (data obtained from *ClinicalTrials.gov*). Additionally, protein-based vaccines for corona infection are designed on the basis of full-length Spike, S1, RDB nucleocapsid, formulated with various other adjuvants (**Table 1**). Even the question of adjuvant side-effects, not specifically related to COVID-19 vaccines, is global risk issue and must not be ignored. By the time of writing this article it is registered 70 studies dealing with protein based vaccines, among which 55 are in pre-clinical phase, and 15 are in clinical phase of research all with different adjuvants **(Figure 1, Table 1).**

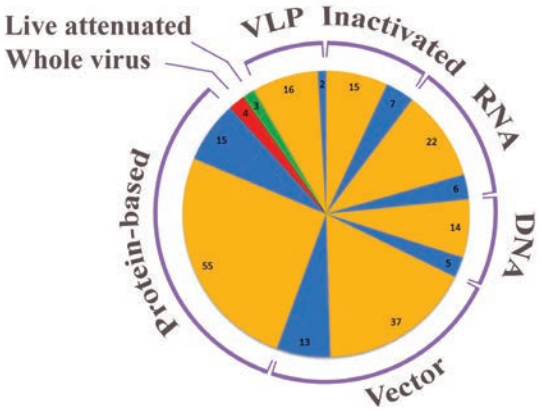

Figure 1. Types of COVID-19 vaccines. The figure shows 8 types of vaccines (inactivated, RNA based, DNA based, produced in vector, protein based, whole virus, live attenuated and VLP) which are in progress. Blue portion of the figure represent the studies which are in pre-clinical phase, and blue represent clinical trials. Data were obtained from Draft landscape of COVID-19 candidate vaccines by 12 November 2020 from the official website of WHO.

Table 1. COVID-19 vaccines in pre-clinical/clinical phase of testing. Data obtained from WHO website.

COVID-19 vaccine platform	Type of candidate vaccine	Developer/manufacturer	Current stage of evaluation [10]
Inactivated	Inactivated	Sinovac	Clinical – phase 1/2, phase 3
Inactivated	Inactivated	Wuhan Institute of Biological Products/Sinopharm	Clinical – phase 1/2, phase 3
Inactivated	Inactivated	Beijing Institute of Biological Products/Sinopharm	Clinical – phase 1/2, phase 3
Inactivated	Whole-Virion Inactivated	Bharat Biotech	Clinical – phase 1/2, phase 3
Inactivated	Inactivated	Institute of Medical Biology, Chinese Academy of Medical Sciences	Clinical – phase 1, phase 1/2
Inactivated	Inactivated	Research Institute for Biological Safety Problems, Rep of Kazakhstan	Clinical – phase 1, phase 1/2
Inactivated	Inactivated	Beijing Minhai Biotechnology Co., Ltd.	Clinical – phase 1, phase 1/2
Inactivated	Inactivated + Al	Shifa Pharmed	Pre-clinical
Inactivated	Inactivated	Milad Pharmaceutics Co.	Pre-clinical
Inactivated	Inactivated	Zista Kian Azma Co.	Pre-clinical
Inactivated	Inactivated	Kocak Farma Ilac ve Kimya San. A.S.	Pre-clinical
Inactivated	Egg-based, inactivated, whole chimeric virus	Institute of Vaccines and Medical Biologicals (IVAC; Vietnam) / Dynavax / PATH	Pre-clinical
Inactivated	Egg-based, inactivated, whole chimeric virus	Government Pharmaceutical Organization (GPO; Thailand) / Dynavax / PATH	Pre-clinical
Inactivated	Egg-based, inactivated, whole chimeric virus	Institute Butantan (Brazil) / Dynavax / PATH	Pre-clinical
Inactivated	Inactivated + Al	KM Biologics	Pre-clinical
Inactivated	Inactivated	Selcuk University	Pre-clinical
Inactivated	Inactivated	Erciyes University	Pre-clinical
Inactivated	Inactivated whole virus	National Research Centre, Egypt	Pre-clinical
Inactivated	TBD	Osaka University/ BIKEN/ NIBIOHN	Pre-clinical
Inactivated	Inactivated + CpG 1018	Sinovac/Dynavax	Pre-clinical
Inactivated	Inactivated + CpG 1018	Valneva/Dynavax	Pre-clinical
Live attenuated virus	Codon deoptimized live attenuated vaccines	Mehmet Ali Aydinlar University / Acıbadem Labmed Health Services A.S.	Pre-clinical
Live attenuated virus	Codon deoptimized live attenuated vaccines	Codagenix/Serum Institute of India	Pre-clinical

Live attenuated virus	Codon deoptimized live attenuated vaccines	Indian Immunologicals Ltd/Griffith University	Pre-clinical
Non-Replicating Viral Vector	Adenovirus Type 5 Vector	CanSino Biological Inc./Beijing Institute of Biotechnology	Clinical – phase 1, phase 2, phase 3
Non-Replicating Viral Vector	Adeno-based (rAd26-S+rAd5-S)	Gamaleya Research Institute	Clinical – phase 1/2, phase 2, phase 3
Non-Replicating Viral Vector	Adenovirus Type 26 vector	Janssen Pharmaceutical Companies	Clinical – phase 1/2, phase 2, phase 3
Replicating Viral Vector	Intranasal flu-based-RBD	Beijing Wantai Biological Pharmacy/ Xiamen University	Clinical – phase 1, phase 2
Replicating Viral Vector	VSV-S	Israel Institute for Biological Research	Clinical – phase 1/2
Non-Replicating Viral Vector	hAd5 S+N 2nd Generation Human Adenovirus Type 5 Vector	ImmunityBio, Inc. & NantKwest Inc.	Clinical – phase 1
Non-Replicating Viral Vector	Replication defective Simian Adenovirus (GRAd) encoding S	ReiThera/LEUKOCARE/Univercells	Clinical – phase 1
Non-Replicating Viral Vector	Ad5-nCoV	CanSino Biological Inc/Institute of Biotechnology, Academy of Military Medical Sciences, PLA of China	Clinical – phase 1
Non-Replicating Viral Vector	Ad5 adjuvanted Oral Vaccine platform	Vaxart	Clinical – phase 1
Non-Replicating Viral Vector	MVA-SARS-2-S	Ludwig-Maximilians - University of Munich	Clinical – phase 1
Replicating Viral Vector	Replication-competent VSV	Merck Sharp & Dohme/IAVI	Clinical – phase 1
Replicating Viral Vector	Measles-vector based	Institute Pasteur/Themis/Univ. of Pittsburg CVR/Merck Sharp & Dohme	Clinical – phase 1
Non-Replicating Viral Vector	Ad 5 vector for intranasal administration	University of Helsinki & University of Eastern Finland	Pre-clinical
Non-Replicating Viral Vector	Adenovirus Type 5 Vector	Globe Biotech Limited, Bangladesh	Pre-clinical
Non-Replicating Viral Vector	Sendai virus vector	ID Pharma	Pre-clinical
Non-Replicating Viral Vector	Adenovirus-based	Ankara University	Pre-clinical
Non-Replicating Viral Vector	Adeno-associated virus vector (AAV-COVID)	Massachusetts Eye and Ear/Massachusetts General Hospital/AveXis	Pre-clinical
Non-Replicating Viral Vector	MVA encoded VLP	GeoVax/BravoVax	Pre-clinical
Non-Replicating Viral Vector	MVA-S encoded	DZIF – German Center for Infection Research/ IDT Biologika GmbH	Pre-clinical
Non-Replicating Viral Vector	MVA-S	IDIBAPS-Hospital Clinic, Spain	Pre-clinical

Non-Replicating Viral Vector	adenovirus-based NasoVAX expressing SARS2-CoV spike protein	Altimmune	Pre-clinical
Non-Replicating Viral Vector	Adeno5-based	Erciyes University	Pre-clinical
Non-Replicating Viral Vector	Ad5 S (GREVAX™ platform)	Greffex	Pre-clinical
Non-Replicating Viral Vector	Oral Ad5 S	Stabilitech Biopharma Ltd	Pre-clinical
Non-Replicating Viral Vector	adenovirus-based + HLA-matched peptides	Valo Therapeutics Ltd	Pre-clinical
Non-Replicating Viral Vector		Vaxart	Pre-clinical
Non-Replicating Viral Vector	MVA expressing structural proteins	Centro Nacional Biotecnología (CNB-CSIC), Spain	Pre-clinical
Non-Replicating Viral Vector	parainfluenza virus 5 (PIV5)-based vaccine expressing the spike protein	University of Georgia/University of Iowa	Pre-clinical
Non-Replicating Viral Vector	Recombinant deactivated rabies virus containing S1	Bharat Biotech/Thomas Jefferson University	Pre-clinical
Non-Replicating Viral Vector	Influenza A H1N1 vector	National Research Centre, Egypt	Pre-clinical
Non-Replicating Viral Vector	Newcastle disease virus expressing S	Icahn School of Medicine at Mount Sinai	Pre-clinical
Replicating Bacteria Vector	Oral Salmonella enteritidis (3934Vac) based protein expression system of RBD	Farmacológicos Veterinarios SAC (FARVET SAC) / Universidad Peruana Cayetano Heredia (UPCH)	Pre-clinical
Replicating Viral Vector	Intranasal Newcastle disease virus vector (rNDV-FARVET) expressing RBD	Farmacológicos Veterinarios SAC (FARVET SAC) / Universidad Peruana Cayetano Heredia (UPCH)	Pre-clinical
Replicating Viral Vector	YF17D Vector	KU Leuven	Pre-clinical
Replicating Viral Vector	Measles Vector	Cadila Healthcare Limited	Pre-clinical
Replicating Viral Vector	Measles Vector	FBRI SRC VB VECTOR, Rospotrebnadzor, Koltsovo	Pre-clinical
Replicating Viral Vector	Measles Virus (S, N targets)	DZIF – German Center for Infection Research/ CanVirex AG	Pre-clinical
Replicating Viral Vector	Horsepox vector expressing S protein	Tonix Pharma/Southern Research	Pre-clinical

Replicating Viral Vector	Live viral vectored vaccine based on attenuated influenza virus backbone (intranasal)	BiOCAD and IEM	Pre-clinical
Replicating Viral Vector	Recombinant vaccine based on Influenza A virus, for the prevention of COVID-19 (intranasal)	FBRI SRC VB VECTOR, Rospotrebnadzor, Koltsovo	Pre-clinical
Replicating Viral Vector	Attenuated Influenza expressing an antigenic portion of the Spike protein	Fundação Oswaldo Cruz and Instituto Butantan	Pre-clinical
Replicating Viral Vector	Influenza vector expressing RBD	University of Hong Kong	Pre-clinical
Replicating Viral Vector	Replicating VSV vector-based DC-targeting	University of Manitoba	Pre-clinical
Replicating Viral Vector	VSV-S	University of Western Ontario	Pre-clinical
Replicating Viral Vector	VSV-S	Aurobindo	Pre-clinical
Replicating Viral Vector	VSV vector	FBRI SRC VB VECTOR, Rospotrebnadzor, Koltsovo	Pre-clinical
Replicating Viral Vector	M2-deficient single replication (M2SR) influenza vector	UW–Madison/FluGen/Bharat Biotech	Pre-clinical
Replicating Viral Vector	Newcastle disease virus vector (NDV-SARS-CoV-2/Spike)	Intravacc/ Wageningen Bioveterinary Research/ Utrecht Univ.	Pre-clinical
Replicating Viral Vector	Avian paramyxovirus vector (APMV)	The Lancaster University, UK	Pre-clinical
Protein based	Full length recombinant SARS CoV-2 glycoprotein nanoparticle vaccine adjuvanted with Matrix M	Novavax	Clinical – phase 1/2, phase 2b, phase 3
Protein based	Adjuvanted recombinant protein (RBD-Dimer)	Anhui Zhifei Longcom Biopharmaceutical/ Institute of Microbiology, Chinese Academy of Sciences	Clinical – phase 1, phase 1/2, phase 2
Protein based	RBD-based	Kentucky Bioprocessing, Inc	Clinical – phase 1/2
Protein based	S protein (baculovirus production)	Sanofi Pasteur/GSK	Clinical – phase 1/2
Protein based	Adjuvanted protein subunit (RBD)	Biological E Ltd	Clinical – phase 1/2
Protein based	Native like Trimeric subunit Spike Protein vaccine	Clover Biopharmaceuticals Inc./GSK/Dynavax	Clinical – phase 1

Protein based	Recombinant spike protein with Advax™ adjuvant	Vaxine Pty Ltd/Medytox	Clinical – phase 1
Protein based	Molecular clamp stabilized Spike protein with MF59 adjuvant	University of Queensland/CSL/Seqirus	Clinical – phase 1
Protein based	S-2P protein + CpG 1018	Medigen Vaccine Biologics Corporation/NIAID/ Dynavax	Clinical – phase 1
Protein based	rRBD produced in CHO-cell chemically conjugate to tetanus toxoid	Instituto Finlay de Vacunas, Cuba	Clinical – phase 1
Protein based	RBD + Adjuvant	Instituto Finlay de Vacunas, Cuba	Clinical – phase 1
Protein based	Peptide	FBRI SRC VB VECTOR, Rospotrebnadzor, Koltsovo	Clinical – phase 1
Protein based	RBD (baculovirus production expressed in Sf9 cells)	West China Hospital, Sichuan University	Clinical – phase 1
Protein based	SARS-CoV-2 HLA-DR peptides	University Hospital Tuebingen	Clinical – phase 1
Protein based	Multitope peptide-based S1-RBD-protein vaccine	COVAXX / United Biomedical Inc. Asia	Clinical – phase 1
Protein based	Recombinant spike with adjuvant	Iran	Pre-clinical
Protein based	Recombinant S protein produced in BEVS	Tampere University	Pre-clinical
Protein based	RBD protein delivered in mannose-conjugated chitosan nanoparticle	Ohio State University / Kazakh National Agrarian University	Pre-clinical
Protein based	Recombinant spike protein with Essai O/W 1849101 adjuvant	Kazakh National Agrarian University	Pre-clinical
Protein based	Peptides	Neo7Logic	Pre-clinical
Protein based	Recombinant spike protein with Essai O/W 1849101 adjuvant	Kazakh National Agrarian University, Kazakhstan / National Scientific Center for Especially Dangerous Infections	Pre-clinical
Protein based	Recombinant S protein	Max-Planck-Institute of Colloids and Interfaces	Pre-clinical
Protein based	RBD protein (baculovirus production) + FAR-Squalene adjuvant	Farmacológicos Veterinarios SAC (FARVET SAC) / Universidad Peruana Cayetano Heredia (UPCH)	Pre-clinical
Protein based	Protein Subunit	Research Institute for Biological Safety Problems, Rep of Kazakhstan	Pre-clinical
Protein based	RBD-protein	Mynvax	Pre-clinical

Protein based	Recombinant S protein	Izmir Biomedicine and Genome Center	Pre-clinical
Protein based	Peptide + novel adjuvant	Bogazici University	Pre-clinical
Protein based	S subunit intra-nasal liposomal formulation with GLA/3M052 adjs.	University of Virginia	Pre-clinical
Protein based	S-Protein (Subunit) + Adjuvant, E coli based Expression	Helix Biogen Consult, Ogbomoso & Trinity Im-monoefficient Laboratory, Ogbomoso, Oyo State, Nigeria.	Pre-clinical
Protein based	Protein Subunit S,N,M&S1 protein	National Research Centre, Egypt	Pre-clinical
Protein based	Protein Subunit	University of San Martin and CONICET, Argen-tina	Pre-clinical
Protein based	RBD protein fused with Fc of IgG + Adj.	Chulalongkorn University/GPO, Thailand	Pre-clinical
Protein based	Capsid-like Particle	AdaptVac (PREVENT-nCoV consortium)	Pre-clinical
Protein based	Drosophila S2 insect cell expression sys-tem VLPs	ExpreS2ion	Pre-clinical
Protein based	Peptide antigens formulated in LNP	IMV Inc	Pre-clinical
Protein based	S protein	WRAIR/USAMRIID	Pre-clinical
Protein based	S protein +Adjuvant	National Institute of Infectious Disease, Japan/ Shionogi/UMN Pharma	Pre-clinical
Protein based	VLP-recombinant protein + Adjuvant	Osaka University/ BIKEN/ National Institutes of Biomedical Innovation, Japan	Pre-clinical
Protein based	microneedle arrays S1 subunit	Univ. of Pittsburgh	Pre-clinical
Protein based	Peptide	Vaxil Bio	Pre-clinical
Protein based	Peptide	Flow Pharma Inc	Pre-clinical
Protein based	S protein	AJ Vaccines	Pre-clinical
Protein based	Ii-Key peptide	Generex/EpiVax	Pre-clinical
Protein based	S protein	EpiVax/Univ. of Georgia	Pre-clinical
Protein based	Protein Subunit EPV-CoV-19	EpiVax	Pre-clinical
Protein based	gp-96 backbone	Heat Biologics/Univ. Of Miami	Pre-clinical
Protein based	Subunit vaccine	FBRI SRC VB VECTOR, Rospotrebnadzor, Kolt-sovo	Pre-clinical
Protein based	S1 or RBD protein	Baylor College of Medicine	Pre-clinical
Protein based	Subunit protein, plant produced	iBio/CC-Pharming	Pre-clinical

Protein based	Recombinant protein, nanoparticles (based on S-protein and other epitopes)	Saint-Petersburg scientific research institute of vaccines and serums	Pre-clinical
Protein based	COVID-19 XWG-03 truncated S (spike) proteins	Innovax/Xiamen Univ./GSK	Pre-clinical
Protein based	Adjuvanted microsphere peptide	VIDO-InterVac, University of Saskatchewan	Pre-clinical
Protein based	Synthetic Long Peptide Vaccine candidate for S and M proteins	OncoGen	Pre-clinical
Protein based	Oral E. coli-based protein expression system of S and N proteins	MIGAL Galilee Research Institute	Pre-clinical
Protein based	Nanoparticle vaccine	LakePharma, Inc.	Pre-clinical
Protein based	Plant-based subunit (RBD-Fc + Adjuvant)	Baiya Phytopharm/ Chula Vaccine Research Center	Pre-clinical
Protein based	OMV-based vaccine	Quadram Institute Biosciences	Pre-clinical
Protein based	structurally modified spherical particles of the tobacco mosaic virus (TMV)	Lomonosov Moscow State University	Pre-clinical
Protein based	Spike-based	University of Alberta	Pre-clinical
Protein based	Recombinant S1-Fc fusion protein	AnyGo Technology	Pre-clinical
Protein based	Recombinant protein	Yisheng Biopharma	Pre-clinical
Protein based	Recombinant S protein in IC-BEVS	Vabiotech	Pre-clinical
Protein based	Orally delivered, heat stable subunit	Applied Biotechnology Institute, Inc.	Pre-clinical
Protein based	Peptides derived from Spike protein	Axon Neuroscience SE	Pre-clinical
Protein based	Protein Subunit	MOGAM Institute for Biomedical Research, GC Pharma	Pre-clinical
Protein based	RBD-based	Neovii/Tel Aviv University	Pre-clinical
Protein based	Outer Membrane Vesicle (OMV)-subunit	Intravacc/Epivax	Pre-clinical
Protein based	Outer Membrane Vesicle(OMV)-peptide	Intravacc/Epivax	Pre-clinical

Protein based	Spike-based (epitope screening)	ImmunoPrecise/LiteVax BV	Pre-clinical
VLP	RBD-HBsAg VLPs	SpyBiotech/Serum Institute of India	Clinical – phase 1/2
VLP	Plant-derived VLP adjuvanted with GSK or Dynavax adjs.	Medicago Inc.	Clinical – phase 1
VLP	Plant derived VLP	Shiraz University	Pre-clinical
VLP	VLPs produced in BEVS	Tampere University	Pre-clinical
VLP	VLP	Max Planck Institute for Dynamics of Complex Technical Systems	Pre-clinical
VLP	Virus-like particle-based Dendritic Cell(DC)-targeting vaccine	University of Manitoba	Pre-clinical
VLP	VLP	Bezmialem Vakif University	Pre-clinical
VLP	VLP	Middle East Technical University	Pre-clinical
VLP	Enveloped Virus-Like Particle (eVLP)	VBI Vaccines Inc.	Pre-clinical
VLP	S protein integrated in HIV VLPs	IrsiCaixa AIDS Research/IRTA-CReSA/Barcelona Supercomputing Centre/Grifols	Pre-clinical
VLP	VLP + Adjuvant	Mahidol University/ The Government Pharmaceutical Organization (GPO)/Siriraj Hospital	Pre-clinical
VLP	Virus-like particles, lentivirus and baculovirus vehicles	Navarrabiomed, Oncoimmunology group	Pre-clinical
VLP	Virus-like particle, based on RBD displayed on virus-like particles	Saiba GmbH	Pre-clinical
VLP	ADDomerTM multi-epitope display	Imophoron Ltd and Bristol University's Max Planck Centre	Pre-clinical
VLP	Unknown	Doherty Institute	Pre-clinical
VLP	VLP	OSIVAX	Pre-clinical
VLP	eVLP	ARTES Biotechnology	Pre-clinical
VLP	VLPs peptides/ whole virus	Univ. of Sao Paulo	Pre-clinical
DNA	DNA plasmid vaccine with electroporation	Inovio Pharmaceuticals/ International Vaccine Institute	Clinical – phase 1/2
DNA	DNA plasmid vaccine + Adjuvant	Osaka University/ AnGes/ Takara Bio	Clinical – phase 1/2
DNA	DNA plasmid vaccine	Cadila Healthcare Limited	Clinical – phase 1/2
DNA	DNA Vaccine (GX-19)	Genexine Consortium	Clinical – phase 1/2
DNA	bacTRL-Spike	Symvivo	Clinical – phase 1

DNA	DNA plasmids containing S-gene	Biosun Pharmed	Pre-clinical
DNA	DNA plasmid vaccine	Globe Biotech Limited, Bangladesh	Pre-clinical
DNA	Plasmid DNA, nanostructured RBD	National institute of Chemistry, Slovenia	Pre-clinical
DNA	DNA, engineered vaccine inserts compatible with multiple delivery systems	DIOSynVax Ltd / University of Cambridge	Pre-clinical
DNA	DNA vaccine	Ege University	Pre-clinical
DNA	DNA plasmid vaccine RBD&N	Scancell/University of Nottingham/ Nottingham Trent University	Pre-clinical
DNA	DNA plasmid vaccine S,S1,S2,RBD &N	National Research Centre, Egypt	Pre-clinical
DNA	DNA with electroporation	Karolinska Institute / Cobra Biologics (OPENCORONA Project)	Pre-clinical
DNA	DNA with electroporation	Chula Vaccine Research Center	Pre-clinical
DNA	DNA	Takis/Applied DNA Sciences/Evvivax	Pre-clinical
DNA	Plasmid DNA, Needle-Free Delivery	Immunomic Therapeutics, Inc./EpiVax, Inc./ PharmaJet	Pre-clinical
DNA	DNA vaccine	BioNet Asia	Pre-clinical
DNA	msDNA vaccine	Mediphage Bioceuticals/University of Waterloo	Pre-clinical
DNA	DNA vaccine	Entos Pharmaceuticals	Pre-clinical
RNA	LNP-encapsulated mRNA	Moderna/NIAID	Clinical – phase 1, phase 2, phase 3
RNA	3 LNP-mRNAs	BioNTech/Fosun Pharma/Pfizer	Clinical – phase 1, phase 1/2
RNA	mRNA	Curevac	Clinical – phase 1, phase 2
RNA	mRNA	Arcturus/Duke-NUS	Clinical – phase 1/2
RNA	LNP-nCoVsaRNA	Imperial College London	Clinical – phase 1
RNA	mRNA	People's Liberation Army (PLA) Academy of Military Sciences/Walvax Biotech.	Clinical – phase 1
RNA	mRNA	Providence Therapeutics	Pre-clinical
RNA	mRNA	Cell Tech Pharmed	Pre-clinical
RNA	mRNA	ReNAP Co.	Pre-clinical
RNA	D614G variant LNP-encapsulated mRNA	Globe Biotech Ltd	Pre-clinical
RNA	saRNA formulated in a NLC	Infectious Disease Research Institute/ Amyris, Inc.	Pre-clinical
RNA	LNP-encapsulated mRNA encoding S	Max-Planck-Institute of Colloids and Interfaces	Pre-clinical
RNA	Self-amplifying RNA	Gennova	Pre-clinical
RNA	mRNA	Selcuk University	Pre-clinical

RNA	LNP-mRNA	Translate Bio/Sanofi Pasteur	Pre-clinical
RNA	LNP-mRNA	CanSino Biologics/Precision NanoSystems	Pre-clinical
RNA	LNP-encapsulated mRNA cocktail encoding VLP	Fudan University/ Shanghai JiaoTong University/RNACure Biopharma	Pre-clinical
RNA	LNP-encapsulated mRNA encoding RBD	Fudan University/ Shanghai JiaoTong University/RNACure Biopharma	Pre-clinical
RNA	Replicating Defective SARS-CoV-2 derived RNAs	Centro Nacional Biotecnología (CNB-CSIC), Spain	Pre-clinical
RNA	LNP-encapsulated mRNA	University of Tokyo/ Daiichi-Sankyo	Pre-clinical
RNA	Liposome-encapsulated mRNA	BIOCAD	Pre-clinical
RNA	Several mRNA candidates	RNAimmune, Inc.	Pre-clinical
RNA	mRNA	FBRI SRC VB VECTOR, Rospotrebnadzor, Koltsovo	Pre-clinical
RNA	mRNA	China CDC/Tongji University/Stermina	Pre-clinical
RNA	LNP-mRNA	Chula Vaccine Research Center/University of Pennsylvania	Pre-clinical
RNA	mRNA in an intranasal delivery system	eTheRNA	Pre-clinical
RNA	mRNA	Greenlight Biosciences	Pre-clinical
RNA	mRNA	IDIBAPS-Hospital Clinic, Spain	Pre-clinical
T-cell based	CD8 T cell peptide targeting (S, M, N) and (NSPs) SARS-CoV-2 proteins	OSE immunotherapeutics	Pre-clinical

DNA VACCINES

One of the mostly examined vaccines in the last decade is DNA vaccine. The DNA vaccine is based on the principle of synthesis of the DNA sequence in the form of a plasmid encoding a protein antigen of the virus. Under optimal conditions, when this sequence reaches the nucleus, this information is transcribed into mRNA, followed by translation into a protein form, which further leads to activation of the immune system and the formation of antibodies of interest, which would provide a specific immunity [31]. However, this sequence of events has a number of obstacles that need to be solved prior to their application. By the synthesis of the appropriate DNA sequence for vaccine, several items should be enabled: successful transport into cells, resistance to the enzymatic degradation in the cytosolic medium, protection from the nucleus entry defend system while preventing DNA vaccine incorporation within the host DNA [32]. Apart from mentioned, limited ability to control immune response triggered by this kind of vaccines must be accounted.

In spite of the production of DNA vaccines is very fast, easy to design and manipulate with, as well as that they do not require continuous cold storage [33], the very efficient and sophisticated delivery system to the cells is required, and for that purpose around 37 various stabilizers are used for plasmid DNA vaccine stability [34]. But nevertheless, the stabilizers used in these vaccines are not fully evaluated in terms of their toxicity. This is one of two main problems when it comes to the DNA vaccines. Namely, it is already discussed about the risks related to usage of various adjuvants such as monophosphoryl lipid A, soap-based molecule QS21, squalene oil substance MF59, cyto-

sine-guanin pair CpG, and preservatives like Thimerosal which contain a mercury (Hg), as well as stabilizers such as Al [34]. The second is that there are obvious problems when it comes to the incorporation of the foreign sequence of plasmid DNA into the host DNA. There are approximately 1,000 to 4,000 copies per μg of DNA which persist 6 weeks, and 200 to 400 copies per μg of DNA which persist 6 months after vaccination [35]. This alteration (transfection) must not be neglected because it might affect the normal upstream and/or downstream DNA transcription. Moreover, the fact that DNA tests were mostly done on animals, and very little is known about their effects on humans, as well as obvious concerns about possible integration of plasmid DNA into human genome puts to the fore the ethical question about clinical trials on humans [31]. Furthermore, inadequate immunogenicity of DNA vaccines, pointed out that these vaccines could not be a proper alternative to the conventional [36]. Namely, due to the immune system is slightly but continuously stimulated by the plasmid DNA vaccine to produce antibodies, the outcome might be serious side effects in a form of chronic stimulation of immune response and subsequent persistent inflammation [31]. On the other hand there are studies explaining that DNA vaccines are more efficacious than vaccines based on recombinant proteins [37] and recombinant viruses [38]. Taking all into account, DNA vaccines are weak inducers of immune responses when compared to the inactive or live-attenuated virus vaccines, and various strategies are being developed to improve their poor efficacy [36]. Another serious concern about usage of DNA vaccine is that they could be triggering button for autoimmune responses, and activation of cancer-causing genes [31]. Moreover, there are literatures data indicating that carriers of the latent infections, where the host co-habituated with the pathogen without conflict (disease tolerance) can be the critical for this kind of vaccination, causing the severe forms of disease upon DNA vaccine application [39]. Namely, Taylor *et al*. explain that DNA form of tuberculosis vaccine may be completely safe when it comes to naive individuals, but if this vaccine is administrated among the people with latent infection it can activate infection leading to severe clinical manifestations [39]. Since the human body lives in symbiosis with various bacteria and viruses, some people achieve disease tolerance with strains that are generally pathogenic, which makes the vaccination as the approach for the development of protection among this population questionable. This is especially referred to the vaccination with prolonged stimulation of immune response, such the DNA vaccines are. Except from basic scientific and ethical controversies related to DNA vaccines even more doubtful is their massive use in order to eradicate the disease. Be that as it may, vaccines in a form of DNA have been more and more discussed as alternatives to conventional vaccine approaches [40]. DNA vaccine is also in the phase of testing for COVID-19, and it is based on sequence which decode the full-length spike (S) or S1 subunit, involved in infection initiation [41]. Furthermore, according to the WHO statistics there are currently 19 types of DNA vaccines for COVID-19 among which 14 are in pre-clinical, and 5 are already in clinical phase of testing (**Figure 1**) [10]. Detailed list is given in **Table 1**.

RNA VACCINES

The new form of genetic vaccines is mRNA vaccine. The principle of mRNA-based vaccines relies on their ability to encode antigen (pathogen) into the protein forms using the host ribosomal machinery. Such a protein is supposed to activate a specific immune response without causing the disease development. The first usage of mRNA as a tool for delivering information for protein production was tested on mice 1990s [42]. Only three years later the injection of influenza mRNA was used to induce immune response which resulted in cytotoxic T lymphocyte generation in mice, suggesting that mRNA might be good alternative for vaccine design [43]. mRNA technology is intensively assessed in drug development and several RNA-based drugs were approved by Food and Drug Administration (FDA) for clinical use [44]. Kim *et al* in 2020 implicated that RNA-based drugs are very easy to design and they possess long-lasting effects, but the main disadvantage is that RNA drugs can only target to the liver efficiently but not into other organs [44]. When it comes to the application of this technology to vaccine design, translation from animal to humans introduced the researchers with a lot of side effects, making a serious concern about their usage in development of immune protection [45]. They are mostly connected with mRNA instability, high innate immunogenicity, and inefficient delivery *in vivo* [46]. To overcome instability of RNA, design of self-amplifying RNA is forced [46]. Conventional mRNA-based vaccines encoded the protein of interest become rapidly digested by the cell enzymes in cytoplasm which goes in terms of their poor specific immunogenicity, similarly to DNA vaccine. On the other hand, self-amplifying mRNAs besides target antigen encode the proteins involved in viral replication, enabling their intracellular amplification [47]. From this point, the risks bound to DNA vaccine entering the nucleus to express the gen of interest, are overcome with mRNA vaccines approaches [48]. On the other hand, it is known that endogenous retroviruses (ERVs) derived from outside retroviruses particles comprise up to 5–8% of the human genome [49, 50]. These alterations were inactivated by mutations or the repair DNA mechanisms which are still unknown, protecting the host from the development of cancer.

Moreover, with advances in science in the field of genetics, it has been shown that much of the DNA (98%) although it does not decode protein sequences is not as dysfunctional as previously claimed [51, 52], but it is discovered that within those 98% DNA over 15% encode functional RNA molecules among which 58648 genes were identified as long non-coding RNAs (lncRNAs) parties [53, 54]. At the same time when the era of RNA technologies and their application in disease treatment started, the first data illustrating regulatory role of RNAs in different biological domains were reported. Only few decades ago, RNAs were treated as basically inert macromolecules that exclusively serve to the protein synthesis [55, 56]. Under these circumstances, the ideal concept of mRNA vaccine as a template for the synthesis of relevant fragment with the aim to promote specific immune reaction, without serious side interaction is expected. However, with the discovery of the network of noncoding RNAs and their active principle in regulation of gene expression through RNA/mRNA interactions, cellular homeostasis maintenance and host defence against viral infection, the spectrum of doubts about possible interactions of introduced sequence with this network is opened [57]. Namely, after the infection with RNA of SARS-CoV in mice, more than 1000 lncRNAs were shown to be expressed activating IFN response [58]. Some studies pointing out that to overcome this high innate immunity, in terms to improve the mRNA vaccine, the early type of IFN I must be reduced [59]. To solve these problems two approaches of mRNA design are used to lower degradation of mRNA and to ensure its translation. First is pseudo nucleotides (nucleosid-modified) mRNA, and the second is the vaccine based on optimization of the nucleotide sequence with unmodified nucleoside [60]. But, there are still no data pointing out which vaccine is better for prophylactic use in humans, modified or unmodified mRNA. Additionally, it is well known that viral RNA interacts with host micro (mi)RNAs leading to its post-translational repression avoiding replication in tissue compartments, which is the method of eliciting host immune response. Thus viral RNA accumulates into the nucleus and uses host transfer (t)RNAs as a primer for replication. Such a produced nuclear (nc)RNAs further regulates host cellular function [61]. Based on that, viral RNAs possess mechanisms to manipulate and take over the host lncRNAs function [62]. Furthermore, there is no doubt that there are certain homologies between viral and human RNAs which can result in proteins homolog generation. Such proteins might be inhibitors for intracellular signalling pathways, causing the depletion of physiological statements and impairing the state of metabolic diseases such as diabetes, which is seen on the example of enterovirus RNA [63-65]. These are the risks which must be examined in detail in order to clinical use of such a type of vaccine.

All this underlined possible interactions in intracellular pool of RNA, which were totally unknown half of the century ago and accordingly, excluded from evaluation at the moment of accelerated research in the area of RNA technology in disease treatment. Bypassing these facts, some review articles highlight the commercial aspect of genetic vaccines [66]. Apart from all concerns, mRNA vaccines for COVID-19 are developing very fast, shortening the period of testing [67-76]. In that manner, the references list pointing to COVID-19 mRNA vaccine development is growing (**Table 1**). By now 28 types of COVID-19 vaccines based on mRNA technology are in preparation, among which 22 are in pre-clinical and 6 in clinical phase of testing (**Figure 1**).

In terms of genetic vaccines certain *pros* is that the DNA/RNA fragments do not cause COVID-19 infection, but the *cons* are unpredictable interactions at the level of the genome, RNA regulatory network as well as inability to control dynamic, intensity and lasting of immune response. Attached to the last, exaggerated risks of genetic vaccination for the latent asymptomatic carriers of the virus should be taken into account [35, 77-79].

CONCLUSION

Very soon after the coronavirus was detected in December 2019, genetic sequencing of the COVID-19 genome was performed a month later, which led to an urgent international response for the rapid development of a vaccine. Even that public's confidence to the vaccination is built on the standard "old fashion" procedures in protective immunity development, absolute supremacy in designing and placing COVID-19 vaccines has been attained by new technologies such as those based on mRNA and DNA concepts. So far, both approaches are multiply tested against different viral diseases but neither of them passed the route from clinical trials to application, regarding to not trivial obstacles that have occurred on their way to the market and are not related only to satisfactory efficiency in acquiring the desired immunity. The controversies related to the design as well as potential application of both types of vaccines, independently from the development of immune protection, implied the entry into the field of complex and under-known interactions within the cell, not only just at the genome level as is most commonly speculated. Furthermore, the compensatory mechanisms for maintaining the homeostasis inside human genome, as well as their involvement into many pathological processes are still a mysterious, which demands a lot of investigation before the usage of genetic tools in terms of immunisation.

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COVID-19 kao prekretnica za upotrebu novih vrsta vakcina

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Kratak sadržaj

COVID-19 je infektivna bolest izazvana virusom SARS-CoV-2 koji pripada grupi Coronaviridae. Simptomi bolesti su prvenstveno prisutni u obliku respiratornog sindroma sličnog simptomima do kojih dovode drugi respiratorni virusi poput virusa gripa, kao i virusa koji dovode do oboljenja poznatih pod imenom SARS i MERS. Međutim, na osnovu kratkog kliničkog iskustva, pokazalo se da COVID-19 utiče na sve organske sisteme ali je patogeneza bolesti i dalje nejasna. Blokada u razmeni gasova, sistemska hipoksija i poremećaji u koagulaciji prate najtežu kliničku sliku. Pored toga, ovaj virus pripada grupi RNA virusa koji dele zajedničke karakteristike sa virusom HIV-a, što ga čini veoma izazovnim u pogledu terapije savremenim medicinskim pristupima. Trenutno se širom sveta vode polemike o tome kako se boriti protiv ove epidemije. Pored higijenskih mera i mera bezbednog rastojanja i izolacije, postoji važna polemika oko profilaktičkih vakcina. U ovom preglednom članku pruža se uvid u sve aspekte vakcinacije i metode za njihov dizajn zasnovane na tehnologijama koje poseduje savremena nauka sa posebnim osvrtom na genetske (DNK, RNK) vakcine, i postavlja pitanje da li postoji opravdanost njihove urgentne primene u slučaju pandemije COVID-19.

Ključne reči: *COVID-19; Vakcine; Oslabljeni virusi; VLP; Vektor; Protein; DNK; RNK.*

REVIEW PAPER

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Nutrition for critically ill patients: The role of glutamine in nutritional immunomodulation

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Abstract

Glutamine is a nonessential amino acid, conditionally essential in stressful conditions (catabolism/hyper catabolism). The lungs, liver, brain, and skeletal muscles take part in specific glutamine synthesis pathways. Plasma concentration of this amino acid is about 50% of total amino acid concentration and about 60% of free amino acids in the body. It is a significant source of energy for all cells, including immune cells. Glutamine can be used as a substrate for nucleotide synthesis. In the last few decades, advanced technology significantly changed the treatment of critically ill patients. Mechanical ventilation, blood products transfusion, renal replacement therapy, invasive monitoring, and many other technical procedures prolong the life of patients by changing and modulating homeostasis and developing new pathways and mechanisms of adaptation – allostasis. Systemic inflammatory response and immunomodulatory activity are part of complex underlying mechanisms involved in allostasis. Nutrition is an important part of the strategy for the treatment of critically ill patients. Based on recently published results, few nutrients (omega-3 fatty acids, arginine, glutamine), when added to the standard formula for enteral and parenteral nutrition, reduce intensive care unit (ICU) stay, infection rate, and the duration of mechanical ventilation in critically ill patients. Glutamine has a high immunomodulatory capacity, as fuel for muscles and a "shuttle" for nitrogen, protecting lung and gut function as well as the function of immunocompetent cells. The most vulnerable systems in COVID-19 patients are respiratory and renal. Despite no universally accepted strategy, treatment with glutamine could have an important role in protecting the cellular integrity of immune cells, alveolar-capillary, and enteral membrane.

Key words: *Critically ill patients; Nutrition; Immunomodulation; Glutamine; COVID -19.*

INTRODUCTION

Surgical, non-surgical, and poly traumatized critically ill patients often need prolonged intensive care treatment, Critically ill patients, some of them, surgical and non-surgical as well as poly-traumatized need prolonged intensive care treatment.. This is dependent on the type and extensity of surgery or trauma and individual functional reserve of cardiovascular, respiratory, and other organ systems. During the intraoperative and immediate postoperative period, these patients are exposed to hypotension, hypoperfusion, and hemodilution with subsequent deterioration in tissue perfusion and oxygenation. Eventual hypothermia, excessive blood loss with extensive volume replacement, blood and blood products transfusion with volume distribution misbalance often take part in the complex postoperative period.

Treatment of these patients demands different modalities of respiratory support and mechanical lung ventilation, invasive hemodynamic monitoring [1,2], fluid balance, and a wide spectrum of noninvasive diagnostic and therapeutic procedures. All of these procedures trigger the systemic inflammatory stress response of the organism. Trauma (surgical procedure), mechanical lung ventilation, and different invasive diagnostic and therapeutic procedures, alongside hypoperfusion and hypoxia, considerably increase infection

risk. Immuno-insufficiency with infection is a predilection for the development of sepsis and multiorgan failure. This triggers a series of compensatory mechanisms of allostasis, an endeavor to maintain homeostasis in completely disturbed regulatory mechanisms of critically ill patients, primarily directed to improve and optimize tissue perfusion/oxygenation ratio.

In the early 80s, many new concepts have been introduced in the everyday clinical management of critically ill patients. Girard and Raffin [3] introduced the term chronic critical illness syndrome – CCIS, the concept based on the net of previously unknown compensatory mechanisms, initiated to restore the balance of different levels of neuro-humoral regulators. In chronically critically ill patients, horizontal and vertical synchronization of complex regulatory mechanisms is impaired: hypothalamus–pituitary, corticotropic releasing factor – an adrenocorticotropic hormone, catecholamine, glucagon, growth factor, and vasopressin. Macrophages/monocytes stimulate tumor necrosis factor (TNF-α) secretion. Hyper catabolism and water retention due to hypercorticism as well as peripheral hypothyroidism and resistance to insulin with subsequent glycogenolysis, gluconeogenesis, and lipolysis are part of complex hormonal misbalance. Shoemaker and coworkers, at the same time, emphasized the importance of balanced perfusion and oxygenation in tissue during the management of the critically ill, optimizing oxygen delivery, and consumption [4].

Despite the increased concentration of plasma nutrients during the treatment, its consumption is limited by insulin resistance and lipoprotein lipase inhibition. In parallel, due to the action of cytokines (TNF-α, interleukine-1, interleukine-6) and glucocorticoids, liver function is reprogramed into the direction to produce acute phase reactants (C-reactive protein, fibrinogen, and immunoglobulin).

The critical illness, associated with a broad spectrum of clinical signs, can be divided into four stages regarding the changes during allostasis: acute critical illness, prolonged acute critical illness, chronic critical illness, and recovery from critical illness. The most critical subsets of patients are those in acute (first 3 days) and prolonged acute critical illness (3-10 days of intensive treatment) [5). During the chronic stage of critical illness (after 14 days of intensive treatment), the patient is mechanically ventilated, tracheotomy is performed, and suffers complex disorders with hypoalbuminemia, anasarca, and stress-induced hypoglycemia, vitamin D deficiency and severe polyneuropathy and myopathy induced by critical illness.

Recently it became clear that intensive metabolic support (IMS) represents the cornerstone of CCIS treatment. Consistent blood sugar control, intensive insulin treatment, adequate nutritive treatment based on the knowledge about allostasis and nutritive pharmacology are essential for IMS [5].

NUTRITION FOR CRITICALLY ILL PATIENTS

The old surgical principle "if the bowels work, use them" is still valid today with specific modifications, unless it is limited by the nature of the disease or surgical treatment of esophagus, intestine, or upper respiratory tract burns. The energy demands and uptake depend on the patient's condition, catabolism degree, presence and stage of infection, and inflammatory response. There are many enteral and parenteral nutrition products, standard or adjusted for patients with different conditions such as diabetes, renal, and liver damage. Nutrition of patients is directed towards two treatment goals to achieve the acquired deficiency correction and modulate systemic stress response [6].

Basic principles

It has been known that respiratory muscle work depends on nutrition, electrolyte, and hormonal levels, oxygen transport, and other metabolic factors. Protein catabolism and musculature weakening are often seen in critically ill patients [7]. Regular hypoxic and hypercarbic ventilatory response can be disturbed during starving. On the other side, hyperalimentation can prolong the weaning of mechanical ventilation by excessive CO_2 production. Bicarbonate excretion as a result of hyperventilation (relatively frequent in patients with COPD) can thwart the process of weaning of mechanical ventilation because patients have decreased hypercarbia compensation capacity.

Numerous disorders can be the cause of respiratory muscle dysfunction. Phosphate and magnesium deficiency can cause respiratory muscle weakness and prolonged weaning. Hyperthyroidism, insulin, glucagon, and corticosteroid misbalance may remarkably deteriorate the optimal muscular function and delay early patient mobilization.

There is no consensus in clinical practice: when to start the nutrition and on what regime; what is the optimal caloric intake, and if treatment duration and outcome depend on caloric intake and administration route [8]. There is an impression that it has been accepted that a critically ill patient's daily energetic requirements are 25 -30 kcal/kg/day and that the enteral route of nutrition is the most efficient [9]. Before enteral nutrition (EN) starts, the possibility of ischemic bowel disease and hypoperfusion should be ruled out because food intake during hypotension or inotropic support can trigger bowel ischemia [6]. As a general principle, it has been accepted that the enteral route of nutrition in an unstable patient should be avoided as long as inotropic agents maintain pressure and perfusion hemodynamics.

Total parenteral nutrition (TPN) is widely accepted as part of a critically ill patient's treatment and brings lower morbidity, complication, and mortality rate. Ac-

cording to the American Society for Parenteral and Enteral Nutrition (ASPEN, https://www.nutritioncare.org/Guidelines_and_Clinical_Resources/Clinical_Guidelines/) and European Society for Clinical Nutrition and Metabolism (ESPEN, https://www.espen.org/guidelines-home/ guidelines espen-guidelines) guidelines, EN should be started in the early postoperative stage. In patients with good nutritive status, TPN should not be initiated in the first 5–7 days and if it is not expected to be used for more than 7 days. In patients who require EN in the early postoperative stage, early initiation of immunomodulatory formulas is recommended, especially based on arginine and fish oil [8]. Abunnaja *et al.* recommend early implementation of immunomodulation based on glutamine [11].

NUTRITION AND IMMUNOMODULATION

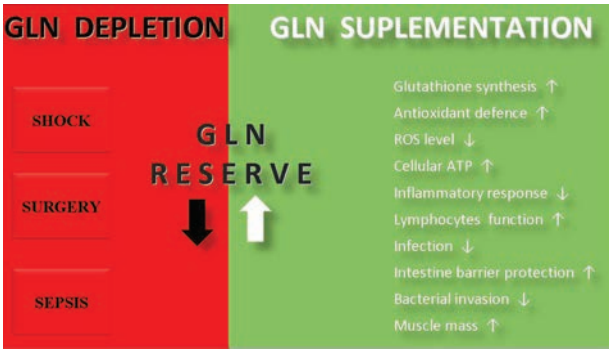
During the last decades, we have been facing more severe cases of intrahospital infections caused by multidrug-resistant bacteria, associated with increased morbidity and mortality rates. Numerous clinical studies have examined the effect of omega-3 fat acid, arginine, glutamine, and nucleotides added to standard nutrition formulas on critically ill patient's treatment outcomes [10]. The authors reported lower infection incidence, shorter general intensive care unit (ICU) and in-hospital stay, shorter mechanical ventilation time in patients treated with nutrients with immunomodulatory potential, and lower treatment expenses.

Immunonutrition and gastrointestinal system

In recent years integrity of the enteral barrier and its protection by enteral nutrition (at least 500 kcal/day) has been requested. In both, liberal and conservative approach, correction of intestine hypoperfusion and hypoxia alongside EN is essential to prevent bacterial translocation and bacterial endotoxin intoxication. If treatment to maintain the enteral barrier is unsuccessful, cytokine and systemic hormones should be activated. The gastrointestinal system is extremely vulnerable to hypoxia and hypoperfusion and has a unique role in the immune response. The small intestine and colon metabolize large amounts of glutamine, where glutamine as a source of energy is more important than glucose. The inflammatory response triggered by surgery results in the realese of inflammatory cytokines (TNF, IL-1, and IL-6), adrenocorticotropic hormone (ACTH), antidiuretic hormone (ADH), catecholamine, and cortisol. Acute response to stress leads to hyper catabolism and skeletal muscle degradation, amino acid mobilization, and an increase of glutamine and alanine levels in the systemic amino acid depot. Critically ill patients usually lose 5-10% of their body mass during one week in the ICU.

Glutamine – essential "nonessential" amino acid

Glutamine has a role in maintaining cellular sodium pump, thereby cellular osmolality. It represents a "source of energy for muscles" and "nitrogen transporter" in the process of muscle regeneration and glycogen depot restoration during the stress, as well as an alternative energy source for the myocardium [12]. The nonessential amino acid that becomes essential in stress conditions is often termed "conditional" essential amino acid. In healthy persons, it is distributed in all tissues (average 70–80 g). It is synthesized from glutamate by glutamine synthetase, mostly in skeletal muscle, lungs, liver, and brain. It constitutes 50% of plasma amino acids (500–800 μM/l) and up to 60% of all body amino acids. Glutamine is a remarkable source of energy during stem cell and some immune cell division [11, 13]. In the central nervous system, it represents the substratum for neurotransmitter (gamma-aminobutyric acid – GABA) synthesis. It has been recently confirmed that astrocytes have a role in the resorption of an excessive amount of glutamine and its recycling out of the synaptic space. Glutamine has an important role in ammonia detoxification and glucose metabolism regulation, alongside glycemia regulation (**Scheme 1**).

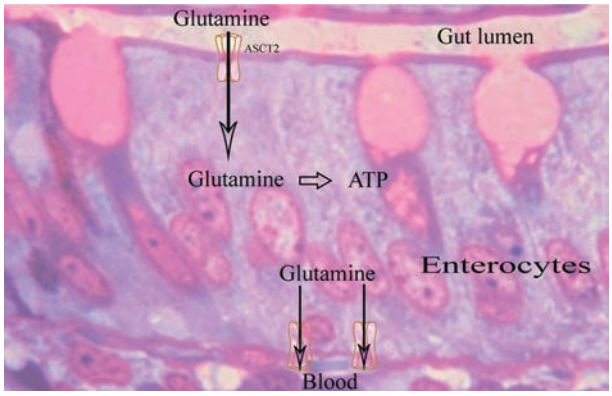


Scheme 1. Potential effects of glutamine supplementation in critically ill patients.

The liver has a special role in glutamine synthesis, as well as in consumption. Glutamine has an important role in stimulating the liver's detoxification role (fatty acid metabolism, chemotherapy, cirrhosis). The absorption capacity of hepatocytes is determined by urea cycle intensity, whereas blood pH and NH_3 metabolism are regulated.

Enterocytes represent a vulnerable glutamine-dependent system as glutamine derived from food intake is the principal energy source for enterocytes and immune cells, initiating underlying mechanisms for their fast regeneration. Based on a great variety of mutually interdependent mechanisms: glutathione stimulation, NO, polyamine, nucleotides, and some amino acids (alanine, citrulline, and proline) synthesis. Protection and stimulation of enteral integrity and stability are among the underlying principles in colitis, Crohn's disease

diarrhea, and chemotherapy-induced colitis treatment by applying Glutamine 20 g/day [13] (**Scheme 2**).



Scheme 2. Glutamine absorbtion in gastrointestinal system.

The extreme effort, infection, stress, and trauma may provoke glutamine deficiency in critically ill patients, thereby deficiency of energy source for many of their cells (lymphocytes, macrophages, and T-cells). Daily substitutional dose in healthy persons is 2-5 g/day, and in severely ill patients (AIDS and bone marrow transplanted) up to 40 g/day [14]. Glutamine neutralizes inflammatory mediators and raises cell immune response and level of anti-inflammatory agents. It is a precursor of glutathione – the most important intracellular antioxidant. Glutamine has a role in intestinal barrier maintenance [15], insulin-mediated utilization of glucose, increased arginine synthesis in kidneys, and increased taurine plasma level (which is vital for cell volume regulation).

CLINICAL EXPERIENCES

Conventional amino acid solutions do not contain glutamine because of its instability in solution and insolubility at high concentrations. Glutamine is synthesized and used in the form of dipeptides L-alanyl-L-glutamine and glycyl-L-glutamine, which are stable in solution and, after intravenous implementation, can be rapidly degraded by hydrolysis in plasma. Despite many publications, debates, and studies, there is no clear consensus. Numerous clinical trials have been conducted with the purpose of examining whether glutamine-rich TPN and EN contribute to better treatment outcomes. A meta-analysis of 355 elective surgery patients treated by glutamine-rich TPN showed a significant reduction in postoperative infection rate and hospital stay [16]. A meta-analysis of 14 randomized control studies that outreached 751 critically ill patients after elective surgery showed that high glutamine doses, more than 0.20 g/kg/day, significantly reduce infection rate, hospital stay, and mortality. In contrast, low doses of glutamine had no effect on those parameters [17]. A randomized control trial was conducted in 20 patients with a head injury (Glasgow coma score 5-12]; divided into two groups: -control group (10 patients) fed by standard enteral formula, and study group fed by a standard enteral formula with the addition of glutamine 30 g/day and probiotic (fermented milk with *Lactobacillus johnsonii*). The number of days on mechanical ventilation was lower in the study group (no statistically significant difference) with a significant reduction in the infection rate and duration of the ICU stay [18].

Numerous studies were conducted in recent years, with various methodologies, inclusion criteria, IMS regime, and glutamine time of administration, dosage, and treatment duration.

Stehle *et al.* conducted a meta-analysis of randomized controlled studies that examined the effect of parenteral nutrition with glutamine dipeptide regime on infection rate, hospital stay, and mortality [19]. Glutamine administration was performed by current guidelines (0, 3-0,5 g/kg/day, up to 30% of nitrogen demands) in combination with adequate nutrients. Sixteen studies were analyzed (842 critically ill patients with multiple conditions: sepsis, secondary peritonitis, severe acute peritonitis, multiple traumas, and severe burns without liver/kidney failure, thermodynamically stable) with no coherent methodological approach in regard to the duration of mechanical ventilation, duration of hospital stay, and ICU mortality. Despite these differences, the authors reported that: those meta-analysis results strongly suggest the benefit of glutamine dipeptide usage as a supplement to parenteral nutrition. Guidelines for glutamine dosage 0, 3-0,5 g/kg/day (it should be less than 30% of daily protein requirement) should be followed. The use of glutamine as a supplement was related to lower infection rates and in-hospital mortality. Studies that compared hospital stay and mechanical ventilation duration, ICU stay duration, and mechanical ventilator support usage concluded that glutamine usage improved these parameters. It has also been shown that glutamine supplementation led to treatment cost reduction [19].

It has also been investigated which route of administration of glutamine, enteral or parenteral, is more beneficial. According to the results, it seems that both routes have some advantages. Parenteral route causes faster effect onset and broader organ outreach. The enteral route had emphasized the local effect on preserving physical and enteral immune system integrity by protecting enterocytes and regional lymphatic-immune system. In comparison, glutamine administrated by enteral route reaches the systemic circulation in smaller doses.

Within the elaborate illness presentation of COVID-19 patients, malnutrition and negative nitrogen balance dominate and thereby impact treatment outcome without coordinated treatment strategy. There is no consensus, but due to recent publications, it is

recommended to use nutritive support as an important part of treatment [20, 21]. The most vulnerable systems in COVID-19 patients are respiratory and renal. Based on the aforementioned clinical experience in CCIS, glutamine could have an important role in the protection and restoration of cellular integrity of the alveolar-capillary and enteral membrane [22, 23]. First, efforts to make protocols and guidelines for COVID-19 patients management and nutrition are made. Algorithms support early enteral nutrition via the gastric route, hypocaloric nutrition in the first 5-7 days, protein delivery of at least 1.2 g/kg/day, while consideration is given to pandemic nutrition resourcing and planning [24]. In a recently published review article, authors concluded that nutritional therapy, together with pharmacological therapy, undoubtedly helps the COVID patient to overcome the acute phase of the disease first and to shorten recovery times [25].

CONCLUSION AND PERSPECTIVE

Survival, proliferation, and function of immune cells in many ways depend on glutamine availability. During catabolic/hypercatabolic processes in critically ill patients, glutamine stores are depleted, decreasing its availability. However, the low glutamine blood level is not always detected in all critically ill patients or patients with metabolic disturbance. There is no unique ground point, but the impression that the level of its availability should individualize glutamine therapy prevails. New studies are needed so we could examine and eventually set the guidelines for this matter. At the same time, there is no consensus about the dosage and regime of administration. On the basis of recent studies, ASPEN, and ESPEN guidelines, the nutritive immunomodulation is based on arginine, glutamine, omega-3 acids (eicosapentaenoic acid – EPA and docosahexaenoic acid – DHA), and omega-6 fatty acids. The most common opinion is that protein recoupment in the early postoperative stage should be 1,2–2,0 g/kg/day with the addition of glutamine 0,3–0,5 g/kg/day or 30% of daily protein requirements.

A recent, unexpected tsunami of coronavirus (COVID-19) pandemic overstrained the healthcare system worldwide. We are seeing an increasing number of patients with complex clinical conditions with a great variety of clinical symptoms, from very mild to severe respiratory, renal, circulatory, and gastrointestinal insufficiency as a consequence of underlying more or less severe immune deficiency. Without consensus in treatment and management worldwide, with frequent changes, sometimes opposite recommendations, intensifies the need to use previous experience in the management of critically ill patients. Based on that experience, immunonutrition based on micronutrients with immunomodulatory capacity might be part of treatment in these patients. So far, there is no consensus regarding dosage, timing, and the most important, early administration of glutamine, besides vitamin C, vitamin D, vitamin E, zinc, and other supportive therapies. Still, some reports, based on initial experience, offer promising results. Clinicians need to tailor the management of COVID-19 patients based on experience with similar multiorgan failure conditions in the critically ill. Time will open new questions and dilemmas before us.

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Ishrana kritično obolelih pacijenata: Uloga glutamina u imunomodulaciji ishranom

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Kratak sadržaj

Glutamin je neesencijalna aminokiselina koja postaje esencijalna u uslovima stresa (katabolizma /hiperkatabolizma) te se često naziva „uslovno" esencijalna aminokiselina. Sintetiše se iz glutamata dejstvom glutamin sintetaze, pre svega u skeletnim mišićima kao i u plućima, jetri i mozgu. Glutamin čini do 50% aminokiselina u plazmi i do 60% slobodnih aminokiselina u telu. Supstrat je za sintezu nukleotida a predstavlja i značajan izvor energije za sve ćelije uključujući imunske ćelije. Tokom poslednjih decenija značajno se promenila strategija lečenja kritično oboleleih. Invazivne dijagnostičke i terapijske procedure kao što su mehanička ventilacija, transfuziona terapija, supstituciona terapija bubrežne funkcije i invazivni hemodinamski monitoring značajno su promenile i produžile život modulirajući mehanizme homeostaze, inicirajući nove forme i mehanizme adaptacije – alostazu. Sistemski inflamatorni odgovor i imunomodulacija su deo komleksnih mehanizama uključenih u alostazu. Vrlo važan deo strategije lečenja kritično obolelih je ishrana. Skorašnja istraživanja pokazuju da neki nutrijenati (omega-3 masne kiseline, arginin, glutamin) kada se dodaju standardnoj formuli za enteralnu i parenteralnu ishranu, doprinose kraćem lečenju kritično obolelih u jedinici intenzivnog lečenja, nižoj učestalosti infekcija i kraćem trajanju mehaničke ventilacije. Glutamin poseduje visok imunomodulatorni kapacit, kao izvor energije za mišiće i „transporter" azota, ima ulogu u protekciji funkcije pluća, creva i imunokompetentnih ćelija. Najosetljiviji sistemi organa kod COVID-19 pacijenata su respiratorni i bubrežni. Iako ne postoji univerzalno prihvaćena strategija za lečenje COVID-19 pacijenata, smarta se da glutamin može imati važnu ulogu u zaštiti integriteta imunskih ćelija kao i enteralne i alveolarno-kapilarne membrane.

Ključne reči: *Kritično oboleli pacijenti; Ishrana; Imunomodulacija; Glutamin; COVID-19.*

REVIEW PAPER

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Virome/SARS-CoV-2 cross-talk: Free space for natural products

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Abstract

One of the most striking marks of infection caused by the SARS-CoV-2 is the distinguishing heterogeneity of the clinical presentations in a population that varies from asymptomatic to severe forms. Pandemic proportion brings into the foreground the number of people with severe forms of the disease, putting aside the fact that a large portion of the population is an asymptomatic, making them silent carriers of the virus. Additional confusion is made by inconsistent data about the presence of the virus in the nasopharyngeal region and the manifestation of the disease symptoms. Different tissue distribution of virus in the body starting from guts, liver, muscles, kidney etc. without any signs of tissue destruction, opens up the possibility that individuals with negative results of PCR test in the nasopharyngeal swab may also be latent carriers of the infection. Overall clinical presentation of the disease is influenced by the initial protection gained through the accumulated "experience" collected from previous encountering of corona family and noncorona viruses, resulting in overlapping of the humoral and cellular immunity. Apart from this, unjustifiably neglected but very significant form of host defense against infection is disease tolerance based on cohabitation with pathogen. It is important to note that consequences of the disease tolerance in terms of pathological and epidemiological aspects are quite different then classical antiviral immune response. This work will elaborate the impact of virome on the course of infection at all stages taking into account both immune resistance, as an ability of the host immune system to eliminate pathogen, and disease tolerance, as a form of host defense with neutral to positive impact to pathogen load. Also, in accordance with the above mentioned, the potential of naturally occurring compounds to profile the course of infection and to support currently available protocols for COVID-19 treatment was discussed.

Key words*: SARS-CoV-2; Disease tolerance; Virome; Immune resistance; Naturally occurring compounds.*

INTRODUCTION

Evidence suggests that about one in five people infected with COVID-19 will experience no symptoms with significantly reduced potential to transmit the virus in comparison to symptomatic one. At the first look paradoxical, some of the studies showed that viral load detected in asymptomatic patients can be similar to that in symptomatic ones, which also theoretically suggests the potential transmission from asymptomatic patients to the rest of population [1]. There is no consensus in science if asymptomatic infections are indeed a 'silent driver' of the pandemic [2]. As part of a large population study in Switzerland, scientists demonstrated viral spread among people living together [3]. Bi *et al*. recently reported that the risk of an asymptomatic person passing the virus to others in their home is about one-quarter of the risks of transmission from a symptomatic person in similar setting [3]. A remarkably lower virus transmission potential of asymptomatic individuals compared to symptomatic ones has been widely confirmed [4, 5]. In parallel, Dr. Ayres *et al*. found that the potential of latent carriers of the pathogen to disseminate infection rapidly decreases through the time counting from the moment of host exposure to the intruder, although the presence and the number of

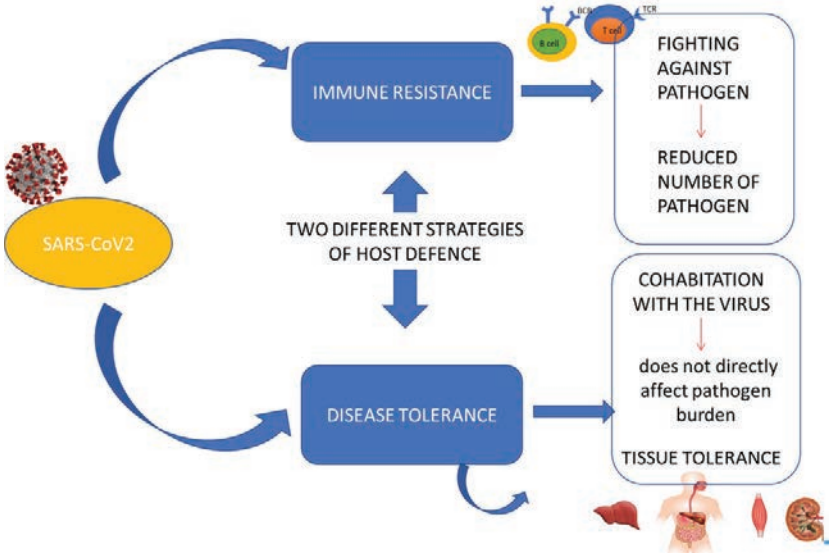
pathogens are similar to those observed in symptomatic individuals [5, 6]. At the first month of pandemic, the rate of asymptomatic infections was estimated as 81% [7]. Lately, numerous meta-analyses evaluated the contribution of asymptomatic individuals in COVID-19 infected population, revealing a remarkable lower range compared to previous reports, approximately from – 15 to 40% [4, 8]. The analysis defined asymptomatic people as those who showed none of the key COVID-19 symptoms during the entire follow-up period. An aspect that has not been taken into consideration and largely relativizes these statistics is the fact that the absence of viral particles in the nasopharyngeal region doesn't mean that the new coronavirus is not present in other tissues, particularly in the gut. There are multiple indications for this. Although it is underlined that SARS-CoV-2 primarily causes lung infection, it was recently reported that SARS-CoV-2 RNA was found in the feces of infected patients [9]. The question arise from this is how many healthy individuals, qualified as negative on PCR test to SARS-CoV-2 are also latent carriers of the virus, whose particles are not detectable in nasopharyngeal area? More importantly, this initiated thoughts about the role of immunity, as well as disease tolerance developed in the past to other members of the coronavirus family and even non-coronaviruses in protection against SARS-CoV-2. How the protection developed upon the introduction of other corona family members contributed to massive asymptomatic or mild clinical presentation of the COVID-19 in the human population? Dr. Patrick *et al*. 15 years ago reported that most sera evaluated for the SARS-CoV-1 antibodies cross-reacted with homologous peptide sequences on HCoV-OC43 nucleocapsid protein, establishing that these cases were indeed producing cross-reacting antibodies [10]. Therefore, it becomes clear that the immune response against SARS-CoV-1 in patients had evolved through repeated infections by different CoVs throughout their lives. This report is further accomplished with the new data confirming a similar overlapping of SARS-CoV-2 cellular immune response with other CoVs and even non-corona viruses, with T cell repertoire recognizing peptides from HCMV, HHV-5 and influenza A virus [11, 12]. T cell mediated fortification collected from the experienced viral infections presented a significant platform for individual protection to COVID-19 infection. Altogether, the host defense against the new virus is orchestrated by whole life experience and the memory of it stored at the virome-host network.

HOST VIROME INVOLVEMENT IN PROTECTION AGAINST SARS-COV-2

Although we usually characterize viruses as pathogens, it has become outward that healthy individuals are also colonized with a vast number of viruses composing the "virome" [13]. Apart from human virome is defined as the total collection of bacterial and eukaryotic viruses in the body, the term virome is often equalized with the gut viral community. The recent investigation of the gut virome population opens a new frame of understanding of the virus commune, shifting it from exclusively pathogenic to intrinsic components of the healthy human gut microbiome with an important role in homeostasis maintenance and host defense. This is a brilliant example of the duality principle in living nature, where any of the elements is not exclusively harmful or beneficial, but flexibly defined within the specific context of interactions. It is obvious that contact between pathogen and host is not exclusively related to risks of disease development, but so importantly connected with health maintenance, with the aim to establish the state of bidirectional comfort. Viral inhabitants of the gut are overall underestimated participants of the microbiota community. While the complex interactions of bacterial and plant viruses in human health maintenance are frequently studied, the presence of mammalian viruses and their relations with other constituents of microbiome as well as input on human health mainly remains unclear [14]. Acute, persistent, and latent viral infections make eukaryotic viruses integrated members of the commensal microorganisms. Norovirus is widespread and it is found in asymptomatic infected humans [15, 16]. Mouse norovirus (MNV) is also found in mice housed in conventional and specific pathogen-free animal facilities [17, 18]. MNV infects immune cells in gut-associated lymphoid tissues and epithelial cells including tuft cells, whereas MNV-infected immunocompetent mice are typically asymptomatic [19]. On the list of eukaryotic viruses detected in human guts as a part of viroma in fecal samples from children were *Adenoviridae*, *Anelloviridae*, *Astroviridae*, and *Picobirnaviridae*, and family members, and species such as enteroviruses, rotaviruses and sapoviruses [13]. Importantly, viruses whose presence is usually connected with disease development such as herpesviruses, polyomaviruses, anelloviruses, adenoviruses, papillomaviruses, polyomaviruses, hepatitis B virus, hepatitis C virus and human immunodeficiency virus (HIV) are also present in the intestinal viromes of some individuals, indicating that the gastrointestinal tract contains viruses known as pathogenic but disassociated from the disease expression (14). While the presence of these pathogenic viruses remain "dormant" in the host, they have become a part of the healthy individual virome specified as "pathobionts" [20, 21]. On the list of pathobionts, even replicative active coronavirus family members (SARS and MERS) were detected in the gut without causing macroscopic or histological changes [22, 23].

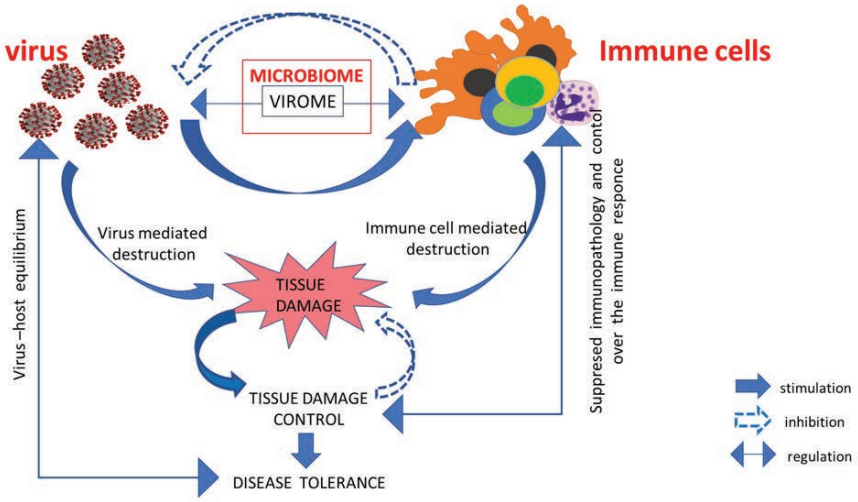
DISEASE TOLERANCE IN COVID-19

There are two possible defense mechanisms performed by the host upon pathogen introduction enabling infected asymptomatic individuals to establish the control up to infection resistance and tolerance (**Scheme 1**) [24]. Traditionally, the defense response in animals against microbes has been equalized to the eradication of microbes through the activation of microbial killing pathways by the immune system, referred as "resistance mechanisms." While the "resistance to infection" is intensively explored through the centuries, starting from Edward Jenner's groundbreaking input to immunization and the final execution of smallpox, Pasteur's germ theory, Robert Koch four criteria, rounded by description of cellular and molecular mechanisms of host defense by Elie Metchnikoff and Paul Ehrlich, the "disease tolerance" is still underestimated in humans [25]. The phenomenon of disease tolerance presents an inherent component of host defense against infections. This approach is based on the tissue damage restriction in the presence and independently from the pathogen load. This can be considered as the opposite of the resistance to infection, the most commonly investigated route used by the host to restore the balance through the reduction of the number of pathogens (**Scheme 1**). Clarification of the distinction between these two defensive mechanisms is of essential importance for understanding the differences in pathological as well as epidemiological consequences of both. Upon pathogen introduction in the host, the buildout of an active immune response will implicate essentially different mechanisms than the development of tissue tolerance, which will be reflected in remarkable differences in disease pathology, host cellular behavior and overall epidemiological signature. It is also important to split the concept of "disease tolerance" from "immune tolerance" [26, 27]. However, distinction between these two processes doesn't exclude their interplay and cooperative contribution of immune tolerance to disease tolerance establishment.



Scheme 1. Two strategies of host deffence against infection. While the immune resistance mechanisms are based on pathogen eradication, disease tollerance presents a nonagressive form of host protection, promoting a host health in parallel with neutral to positive impact on pathogen fitness.

Phenomenon of disease tolerance was first documented in plants in the second half of 19th century by Nathan Augustus Cobb, an American plant pathologist who observed the ability of certain strains to growth regardless of the presence of a fungal infection, describing it as "rust-enduring" not "rust-resistant" wheat [28]. Even though this type of host defense against invaders was discovered and explored in the last century, it was not recognized as important in animals until last decade. Accordingly, disease tolerance can be counted as a completely new field in biology of infection in animals and humans. It was showed that "decision" to fight or tolerate the pathogen following malaria infection can be defined by a genetic variation [29]. Soon after, it was found that the main principle of tissue protection from protozoan-induced hemolysis in mice is provided by the heme-catabolizing enzyme heme oxygenase-1 [29]. The hypothesis that disease tolerance is an ancient form of host protection and health maintenance has been further empowered with the discovery of Dr. Ayres and Dr. Schneider who demonstrated that the simple organisms such as fruit fly *Drosophila melanogaster* can also use disease tolerance as a host defense mechanism in the context of gram-positive and gram-negative bacterial infection [30, 31]. All together, these studies have provided insights into disease tolerance as an alternative and/or complementary form of host defense. Upon pathogen introduction, in interplay with host microbiota, innate, and soon after, adapted immunity are stimulated to restrict pathogen load. In parallel, pathogens alone and/or trapped into the immune system network, promote stress and tissue damage. This enforces activation of tissue damage control mechanisms, implicating a wide range of evolutionary conserved responses to stress and damage. Accurate tissue damage control mechanisms are a leading force of disease tolerance establishment, manifested by functional recovery of parenchyma tissues and vital homeostatic parameters (**Scheme 2**). On the other



Scheme 2. Central role of tissue damage control in immune resistance and disease tolerance orchestration. Intruder alone and/or trapped into the immune system network, promotes stress and tissue damage. This lead to the activation activation of tissue damage control mechanisms. Correct tissue damage control resulted in functional recovery of parenchyma tissues and vital homeostatic parameters. Simultaneously, tissue damage control program buffers immunopathological consequences of triggered antiviral immune response. Optionaly, tissue damage control can lead to virus-host equilibrium and disease tolerance.

hand, tissue damage control programs should enable immune mediated resistance mechanisms to function under limmited immunopathology, leading to successful pathogen clearance and abrogation of disease transmission [26]. In summary, damage control mechanisms play a central role in the host defense profile, orchestrating immune resistance and disease tolerance (**Scheme 2**). In this network, microbiome and more strictly, virome could be a template for host positioning to the upcoming infection. There are clear indications that in the long term period after infection resolution, the virus becomes a member of the viral commune, with less or more replicative potential. It is speculated that its hidden presence within certain organs (e.g., liver, muscles, kidney) apart from the gut where it can be detected, can influence long term immunity. Gaebler *et al*. found continued evolution of the humoral response to SARS-CoV-2 in asymptomatic or mild disease cases between 1.3 and 6.2 months after the infection in a manner consistent with the antigen persistence [32]. Detection in the feces as well as analysis of intestinal biopsies confirmed the presence of whole viral particles in half of the tested individuals [10]. Additionally, there are data confirming good prognostic relevance of the presence of SARS-CoV-2 in guts for disease outcome [33]. This further means that months after the infection in moderate, as well as asymptomatic forms, SARS-CoV-2 became a member of healthy individual virome commune influencing not only dynamics of additional flow in host- virus cohabitation, but through this, affecting the further host contacts with other viruses, both within and out the coronavirus family.

In a certain paradoxical way, the tissue tolerance to SARS-CoV-2 was detected across a wide variety of organs of patients with fatal outcomes of COVID-19 infection, but not as a consequence of the viral load in different tissues [10]. Severe inflammation was restricted to the lungs and reticuloendothelial system, and even there it was not in strict association between inflamed area and the presence of viral RNA or proteins. In different organs such as gastrointestinal tract, heart and muscles, and less often the liver and kidney, frequent presence of SARS-CoV-2 was detected without even minor signs of injury related to it. The authors describe this phenomenon as tissue tolerance toward the SARS-CoV-2. Additionally, tissue inflammation and organ damage in COVID-19 showed an unexpected pattern of inconsistent correlation with the distribution of the viral particles. Lack of strict link between inflammation and virus presence, indicates the possibility that immune cells activated by the viral particles can shift from the viral epitope to self-antigens with a certain homology, leading to autonomies immune attack toward self-tissue.

SILENT CARRIERS OF SARS-COV-2 AND POTENTIAL RISKS FROM THE VACCINATION

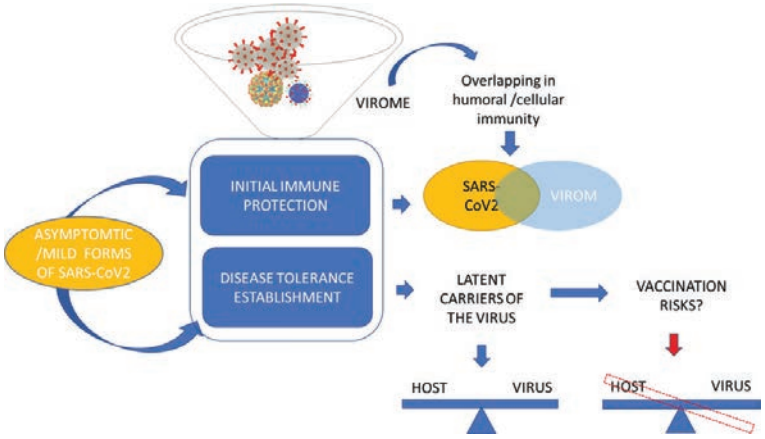
Finally, there are many elements bringing into connection COVID-19 and the infection with *Mycobacterium tuberculosis*. This analogy can be very helpful for better understanding the pathology of the disease as well as the potential risks related to immunotherapeutic approaches in the treatment of COVID-19, preferentially referring to silent carriers of SARS-CoV-2. The contribution of asymptomatic individuals carrying SARS-CoV-2 in the total population under pandemic is remarkable, and our ability to detect them is limited, according to the tissue tolerance platform and random distribution of the virus in tissues. Hypothetically, this group could be extended to the people recovered from the COVID-19 in whose SARS-CoV-2 is retained in guts, and possibly other tissues, in a disease-tolerant manner and in equilibrium with the host under certain control by the host immunity. As it is mentioned above, there are indicators of viral particles persisting out of the respiratory tract after the infection.

At the end of the last century disease induced by *Mycobacterium tuberculosis* was the leading cause of death from infectious diseases [34]. It is estimated that over 2 million of people die from tuberculosis yearly and about 8 million people deal with the disease. Individuals who have been exposed to the bacillus but may have controlled it in the form of a latent infection, may number in hundreds of millions [34].

Taylor *et al*. showed that a plasmid DNA vaccine (Hsp60/lep) that has been previously shown to be highly effective against intravenous or intraperitoneal inoculation with virulent *M. tuberculosis* H37Rv failed to protect mice in an aerosol infection model or in a model of latent tuberculosis in the lungs. Moreover, when the vaccine was given in an immunotherapeutic model, the immunized mice developed classical Koch reactions characterized by multifocal discrete regions of cellular necrosis throughout the lung granulomas [35]. Similar and equally severe reactions were seen in mice inoculated with a vaccine with DNA coding for the Ag85 antigen of *M. tuberculosis*. This previously unanticipated safety problem indicates that DNA vaccines should be used with caution in individuals who may have already been exposed to *M. tuberculosis* [35].

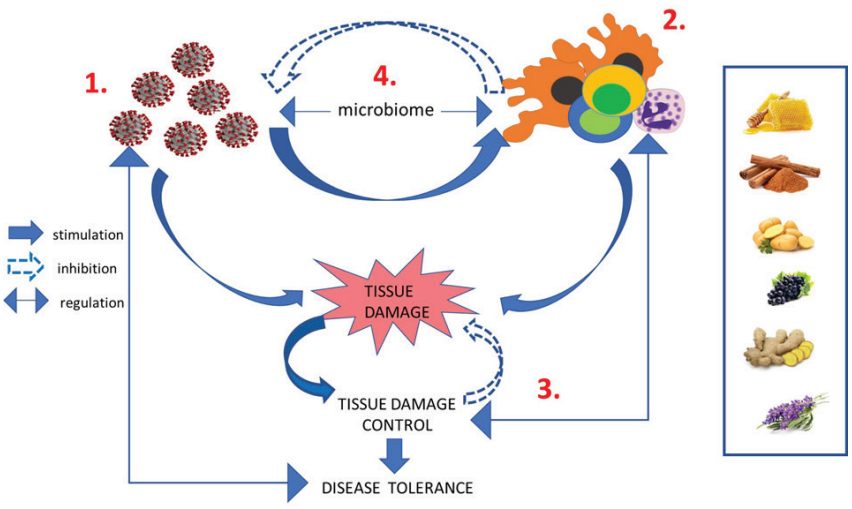
Applying this template on COVID-19 infection, it is reasonable to speculate that a similar approach based on DNA vaccination to protect individuals and support the establishment of collective immunity, could hypothetically lead to development of severe symptoms of the disease, when inoculated into hidden silent carriers of SARS-CoV-2.

In summary, after almost a year of intensive research and collected experience with the patients, there are serious weaknesses in understanding of disease pathology and host defense against COVID-19 infection. A much deeper analysis than a simple reduction to immune resistance and B and T cell-mediated immunity is needed, taking into account the virome-derived initial protection and the development of disease tolerance as a less known, but not less important template of host protection against invaders (**Scheme 3**). From this standpoint, rapidly developed decisions about massive vaccination including the type of vaccines, should be reconsidered (**Scheme 3**).



Scheme 3. Microbiome as a template for the host positioning to the upcoming infection. Apart from classical immune response to infection, disease tolerance, developed through cohabitation between invader and the host, might be important aspect of SARS Cov-2 infection in terms of pathology as well as epidemiology, leading to reevaluation of certain type vaccine usage in latent carriers.

There are several checkpoints defining the flow of the infection (**Scheme 4**). Apart from the intrinsic factors, numerous naturally occurring compounds (NOC) we are exposed to through the diet and beverage, influence each of them, shaping host and pathogen interplay. Most of NOC are active on few checkpoints in parallel, reducing the pathogen viability, promoting establishment of tissue damage control, favoring disease tolerance and/or optimal immune response (**Scheme 4**).



Scheme 4. Naturally occurring compounds on each check point of host defense against infection. Biologically active compounds uptake through food, beverage or supplementation shape the immune response and interfere with conventional therapeutic approaches.

NATURALLY OCCURRING COMPOUNDS IN DEFENSE SERVICE OF COVID-19

At the moment even though vaccination started in some countries we still don't have specific therapy for the management of COVID-19. Thus, a lot of effort is put on the encountering preventive and therapeutic strategies for eradication of this disease. Since the discovery of new drugs is an exhausting and long-lasting process, repurposing and repositioning of currently available drugs is the fastest approach. However, modern medicine is still limited in the treatment of viral infection due to the high viral mutation rate, development of resistance, high amount of side effects and costs of existing therapies [36]. Nowadays only few antiviral drugs on the market are effective enough and one of the possible alternative sources of potentially new drugs is definitely nature. In general, naturally occurring compounds apart from their healing potential serve as matrices for derivatization or inspiration for synthetic drugs made according to their structure when their quantity or delivery is limited. Since 1981 till the mid of 2019, around 40% of approved drugs are isolated naturally occurring compounds or their derivatives [37]. In general, antiviral drugs can be classified into those inhibiting the interaction of the virus with the host cell membrane or receptor, viral uncoating inside the cell, nucleic acid synthesis as the next step in viral life cycle, integration into host cell DNA, proteases, and release of new viruses from the host cell in final instance [38]. Together with these therapeutic approaches directed to different steps of viral infection, other strategies are also settled, targeting cellular receptors or host enzymatic machinery utilized by the virus or modulation of host immune response to viral infection related. Since the pandemic was proclaimed in March this year all the effort is put into the service of COVID-19 eradication. If we switch to SARS-CoV-2 virus, few major proteins, viral and human, enable its inoculation and replication inside the host cells. The penetration of SARS-CoV-2 virus into the host cells happens as a result of the binding of SARS-CoV-2 spike protein (S) to host receptors and on S protein priming by the host cell proteases. Type II transmembrane serine protease (TMPRSS2) cleaves S spike glycoproteins activating the glycoprotein for host cell entry [39]. TMPRSS2 is critical for spreading of other viruses, like influenza A viruses and coronaviruses etc. [40- 42]. Hoffman *et al*. demonstrated that similarly to other coronaviruses, SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) receptor for entring into the host cell and the serine protease TMPRSS2 for S protein priming [43, 44]. ACE2 is an analogue of the angiotensin converting enzyme type I (ACE) important for regulation of blood pressure. SARS-CoV-2 encodes two proteases, the papain-like protease (PLpro) and 3-chymotrypsin-like main protease (3CLpro or Mpro), that are in charge for the proteolytic cleavage of virus polypeptide into nonstructural proteins important for viral replication [45]. The nonstructural proteins further assemble the viral replicase complex, triggering replication and transcription of the viral genome. All proteins mentioned above served as a target for searching the libraries of naturally occurring compounds as potential drugs [46]. Additional support comes from the previous studies on other corona viruses such as SARS-CoV-1 [47]. Important tool in these initial studies is performing a virtual screening of natural compounds libraries using *in silico* molecular docking that gives insights into potential drug candidates. Plenty of *in silico* studies using the molecular targets mentioned above were carried out in the last year. Since many of them are already elaborated elsewhere, only few of them will be presented in this review. One of the first studies was done on traditional Chinese herbs that identified 11 natural products capable of inhibiting ACE2. The bioactive compounds selected in this study were baicalin, scutellarin, hesperetin, nicotianamine, glycyrrhizin, naringin, naringenin, hesperidin, neohesperidin, and nobiletin [48-51]. Further studies highlighted different groups of alkaloids, terpenes, flavonoides, limonoids, lignans, terpenoids, tannins, phenolic acids and fatty acids as compounds of interest. Few hundreds of plants are rich in potential ACE inhibitors and some of them are present in food or spices that are frequently used like cinnamon (*Cinnamomum zeylanicum Blume or Cinnamomum verum J. Presl*.), pepper (*Capsicum* spp.), olive (*Olea europaea* L.), curcumin (*Curcuma longa*), garlic, green tea etc. A second molecular target widely studied is TMPRSS2. Its role in other viral infections like influenza and SARS- CoV-1 is known and inhibitors were found among flavonoids (baicalein and baicalin), terpenes and peptides. Rahman *et al*. segregated 12 metabolites (iridoids, diterpenes and lignans) using *in silico* studies based on TMPRSS2 blockade [52]. The potential TMPRSS2 inhibitors can be extracted not only from plants but also other sources like marine corals, algae, and mushrooms. The third molecular target that attracts attention is 3CLpro, a specific viral enzyme. Gurung *et al*. revealed that terpenoids bonducellpin D and caesalmin B and the flavonoid 5,7-dimethoxyfavanone-40-O-b-d-glucopyranoside showed affinity toward all 3 coronaviruses, SARS-CoV-1, SARS-CoV-2 and MERS-CoV [53]. They are present in some herbs used in Chinese traditional medicine and also in European mistletoe (*Viscum album*). Some other authors indicated potential inhibitors of 3Cpro of SARS-CoV-2 between kaempferol, quercetin, luteolin-7-glucoside, demethoxycurcumin, naringenin, apigenin, oleuropein, catechin, curcumin, and epigallocatechin that can be extracted from lavender (*Lavan-*

dula angustifolia), basil (*Ocimum basilicum*), mandarin (*Citrus reshni*), cinnamon (*Cinnamomum zeylanicum*), chamomile (*Matricaria recutita*), ginger (*Zingiber officinale*), licorice (*Glycyrrhiza uralensis*, *Glycyrrhiza glabra*, and *Glycyrrhiza*), black pepper (*Piper nigrum*), cannabis (*Cannabis sativa*), cloves (*Syzygium aromaticum*), oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*) [46, 47, 49, 53, 54]. It is important to note that the compounds able to block more than one target inside the SARS-CoV-2, might be multiply beneficial. In addition, the fact that some herbal compounds showed activity against other viruses put them into the foreground. For example glycorhizzin was efficient against Human Immunodeficiency Virus Type 1 (HIV-1), Herpes Simplex Virus Type 1(HSV-1), Hepatitis C virus, Varicella-Zoster virus and SARS-Coronavirus. Rhein and chrysophanic acid from *Aloe vera* (*Aloe barbadensis*) and Rhubarb (*Rheum palmatum*) are efficient against poliovirus influenza [55]. It is obvious that naturally occurring compounds are able to interact with the virome mediating the host-pathogen relation in terms of immune resistance as well as disease tolerance as a two forms of host response to intruder. Apart from the fact that human virome includes commensal, pathogenic and novel bacteriophages, eukaryotic viruses involved in acute or persistent infection, endogenous retroviruses and under investigated forms that settle whole organism, it is usually equalised with gut viral family as a part of gut microbiome. Consumption of food and oral admission of pathogens both influence the establishment of host pathogen equilibrium mainly leading to disease tolerance. Trillion of microbes in the human body are actively involved in optimal health maintenance and profiling the host response to infection. Everything that helps maintain the balance in the gut might positively regulate immune response and defense from the disease.

In this term, it is important to mention that a multicenter randomized clinical trial evaluating the efficacy of resistant potato starch, carbohydrate, in reducing the need for hospitalization for COVID-19 positive patients has recently started (NCT04342689) [56]. This study will include 1500 non-hospitalized COVID-19 positive patients. The rationale for this study is that fiber ferment serves as a prebiotic protecting the gut microbiota at a multiple level. Directly, resistant starch feeds the microbiome, decreases ileal and cecal pH promoting the growth of beneficial microorganisms [57, 58]. Protecting the mucus layer, resistant starch prevented the damage of the epithelium and also presented a great support in healing of leaky gut and protecting gut barrier integrity. In addition, it decreases the IL-6 level, one of the mostly abundant inflammatory mediators in COVID-19 patients, by elevating butyrate levels [59]. Butyrate reduces overall inflammation, in particular in lungs, and reduces ACE2 receptor expression, suppressing the entry of viruses. It also induces antimicrobial activity of intestinal macrophages. Beside direct influence on the gut, it influences the function of resident antigen-presenting cells in lungs, weakening the inflammatory reactions [60].

On the other hand, naturally occurring compounds apart from direct antiviral activity have other biological effects that might be important for prevention and also for suppression of the disease. For example, known antioxidative features of naturally occurring compounds will be important in both aspects of restriction of viral reproduction and protection of host cells from virus-mediated damage A huge body of evidence accumulated over the past decade indicates that patients infected with RNA viruses including human influenza virus, Hepatitis C virus (HCV), human immunodeficiency virus (HIV) are under chronic oxidative stress. Reactive oxygen species are important signaling molecules and mediators of essential processes inside the hosts like apoptosis, impaired immune defense and stimulated viral replication [36]. Second important aspect that might be relevant for defense from COVID-19 infection is the immunomodulatory properties of numerous naturally occurring compounds. Apart from their direct effect on the immune system of patients, targeting the viral proteins will have repercussions on disease outcome. Dysregulated inflammatory response is known as a hallmark of COVID-19, and considerable morbidity and mortality is associated with obsessional immune responses and further tissue damage [61]. It is found that SCoV2-PLpro is able to trigger an evasion mechanism against host antiviral immune responses through interferon production due to blocking of IRF3 phosphorylation and nuclear translocation [62]. The other important target that might be influenced by SCoV2-PLpro is NF-kB signaling pathway [63]. Since the viral proteases have the potential to inhibit host innate immune responses and inflammatory response it is reasonable to expect that targeting them with naturally occurring compounds will be a dual therapeutic strategy. The family of naturally occurring compounds provides a source of biologically active molecules that are able to affect COVID-19 infection in all stages, from the initial to the late, and from mild to severe presentations, enforcing different strategies.

NATURALLY OCCURRING COMPOUNDS IN CLINICAL TRIALS

Even though *in silico*, *in vitro* and *in vivo* studies provided a lot of evidence about the potential of naturally occurring compounds against COVID-19 infection, definitive proofs will come from patients. Till today more than 4000 clinical trials focused on COVID-19 were reg-

istered in the US National Library of Medicine Clinical Trials website. Searching the database with COVID-19, phytochemicals, polyphenols, phytotherapy as keywords showed that many of them are intended to explore naturally occurring compounds or extracts as a supplement to therapy or prophylaxis against COVID-19 [56]. Due to the scope of this paper, only a few studies will be presented herein. Few of them are dedicated to Chinese traditional medicine and usually include a mixture of plants and recipes with a lot of empirical data about their efficacy in treatment of viral infections. Prospective, double-blind, randomized trial on 140 COVID-19 patients, evaluating of the effect of dietary supplement of quebracho and chestnut tannins in combination with Vit B12 on cytokines level, and intestinal microbiota composition will be done in Argentina (ClinicalTrials.gov Identifier: NCT04403646). Randomized double-blind placebo-controlled proof-of-concept trial of resveratrol for the outpatient treatment of mild coronavirus disease (COVID-19) will be enrolled on 200 patients with an aim to evaluate the influence of the treatment on the rate of hospitalization (ClinicalTrials.gov Identifier: NCT04400890). Interventional clinical trial on 100 participants is currently ongoing evaluating the efficacy of *Caesalpinia spinosa* extract (P2Et) on reducing the length of hospital stay of patients. The authors suggest that this supplementation will improve the general condition of patients, reduce the inflammatory mediators and the viral load. Interventional study in phase 4 evaluating multiplied therapy zinc, quercetin, bromelain and vitamin C on the clinical outcomes of patients showed that therapy might be useful in prevention of severe presentation of disease [64]. Quercetin is a polyphenolic compound found in onion, red grapes, honey and citrus fruits. It possesses antioxidant, antiviral and anti-inflammatory properties, but also it might inhibit platelet aggregation and capillary permeability [65]. On the other hand, since bromelain, protein-digesting enzyme mixture from the pineapple plant, stimulates natural killer cells and T helper cells, it might be useful as anti-inflammatory agent (ClinicalTrials.gov Identifier: NCT04410510) [66] . Based on the known potential of quercetin to inhibit the production of proinflammatory cytokines and enzymes (cyclooxygenase and lipoxygenase) included in metabolism of arachidonic acid, one more study is dealing with the efficacy of quercetin (Quercetin Phytosome) on the survival time, symptoms and inflammatory parameters of 200 participants (ClinicalTrials.gov Identifier: NCT04578158) [67, 68]. Interventional clinical trial on 524 participants evaluating preventive effect of epigallocatechin-3-gallate (EGCG), a biologically active polyphenol on health care workers that is the most exposed group (ClinicalTrials.gov Identifier: NCT04446065). In another interventional study on 200 patients, safety and effectiveness of dietary supplement of plant polyphenol in conjunction with vitamin D3 will be studied (ClinicalTrials.gov Identifier: NCT04400890). Six registered studies are planned to investigate the potential of honey constituents on COVID-19 infection and health status of patients. From ancient times it is well known that honey and propolis have anti-inflammatory, antibiotic, antifungal, antiviral, antioxidant, anti-cancer, immunomodulatory, hepatoprotective effects and antiviral properties [69-73]. They have been used as supplements for many immune related diseases. The composition of honey varies depending on the plant sources and region where it is collected. A multicenter, placebo-controlled, randomized clinical trial was performed in 4 clinical centers in Pakistan. 313 patients with moderate and severe pathology were included in the study. Patients received honey and *Nigella sativa* seeds in addition to standard therapy [74]. The applied treatment significantly improved clinical signs, viral clearance and survival COVID-19 patients (ClinicalTrials.gov Identifier: NCT04347382). Propolis is composed from 50% resins, 30% waxes, 10% essential oils, 5% pollen, and 5% other compounds like polyphenols and flavonoids [69]. Since it was shown that propolis components have inhibitory effects on the ACE2, TMPRSS2 and PAK1 signaling pathways, a pilot randomized study evaluating the Brazilian green propolis extract on oxygen therapy dependency time or hospitalization time on 120 participants started (ClinicalTrials.gov Identifier: NCT04480593). The initial results of these studies are still awaited. We anticipate that results of some of the mentioned trials will demonstrate the safety and the efficacy of naturally occurring compounds as an adjunctive treatment for COVID-19 infection.

CONCLUSION

COVID-19 infection is characterized by an extremely heterogeneous clinical presentation of the disease, from non-manifestation to severe forms. Today, it is known that the microbiome in a broader sense, or more strictly virome, can serve as a template for the host positioning to the upcoming infection. Recent data confirms that in the long time period upon resolution of infection, virus becomes the integrative part of viral commune, less or more replicative, continuously shaping host response to future exposure to other infections. Apart from classical immune response to infection it is now clear that disease tolerance, developed through cohabitation between invader and the host, might be important aspect of SARS-CoV-2 infection starting from the individual level, in terms of disease pathology, to collective, in terms of epidemiology. This further implicated reevaluation of certain type

vaccine usage in latent carriers. In addition to intrinsic factors such as microbiome, naturally occurring components that we consume through food, beverage or supplementation have a significant role in profiling the immune response and interferes with conventional therapeutic approaches.

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Virom/SARS-CoV2 interakcija: Upražnjeno mesto za prirodne komponente

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Kratak sadržaj

Jedna od najupečatljivijih oznaka infekcije uzrokovane SARS-CoV-2 je heterogenost kliničke slike u populaciji koja varira od asimptomatske do teške forme. Pandemijska srazmera čini da brojnost ljudi sa teškom formom bolesti dolazi do izražaja, potiskujući činjenicu da je veliki deo populacije asimptomatski nosioc virusa. Dodatnu konfuziju unose nekonzistentni podaci o prisustvu virusa u brisu nazofaringealne regije i ispoljavanja bolesti. Različita distribucija virusa u telu, počevši od creva, preko jetre, mišića, bubrega itd., a bez znakova tkivnog oštećenja, otvara mogućnost da osobe sa negativnim rezultatima PCR testa u nazofaringealnom brisu mogu biti latentni nosioci infekcije. U osnovi različite kliničke slike bolesti može biti inicijalna protekcija prema SARS-CoV-2 infekciji stečena akumuliranim „iskustvom" u susretu sa drugim pripadnicima porodice korona ali i nekorona virusa, koja je ishodovala formiranjem humoralnog i celularnog imuniteta sa preklapajućim repertoarom. Osim toga, neopravdano zapostavljeni, ali vrlo značajan oblik odbrane domaćina od infekcije je tolerancija na bolest koja se temelji na suživotu s patogenom. Važno je napomenuti da su posledice tolerancije na bolest u smislu patoloških i epidemioloških aspekata prilično različite od klasičnog imunskog odgovora. Ovaj rad će diskutovati uticaj viroma na tok infekcije u svim fazama uzimajući u obzir oba- klasični imunski odgovor sa ciljem eliminacije patogena i sticanje tolerancije na bolest, kao oblik odbrane domaćina od infekcije u suživotu sa patogenom koji može da varira od neutralnog do sibmiotskog. Takođe, u skladu s gore spomenutim, razmatraće se o potencijal komponenata iz prirode da oblikuju tok infekcije i pruže podršku aktuelnim terapeutskim pristupima u lečenju COVID-19.

Ključne reči: *SARS-CoV-2; Tolerancija na bolest; Virom; Imunski odgovor; Prirodne komponente.*

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Nutritional behavior of students during COVID-19 quarantine

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Abstract

COVID-19 has now been declared a pandemic by the World Health Organization (WHO), and people are under quarantine. During quarantine, continuously hearing or reading about the pandemic can have effects on different aspects of people's lives. One of these very significant effects is on human nutrition. This study aimed to summarize the experiences of the student population in behavior and the quality of nutrition during COVID-19 quarantine with special reference to certain microelements that are necessary for immune responses. Students were invited to do an online survey during May 2020. None of the participants were infected with COVID-19. 34.2% of respondents were under stress due to constant information about the pandemic. A significant number of students had good nutritional habits. Most students did not feel a constant need for food (63.2%), nor did they consume larger amounts of food than usual (67.5%). Students (36.0%) were careful about the nutritional and energy value of food. Most of the students (86.8%) ingested micronutrients mostly through meals. The students had well-balanced meals that had a beneficial effect on their immune responses. Few students (13.2%) have used dietary supplements. Generally, this research may help for a better understanding of the importance of a proper and balanced diet and the use of dietary supplements for maintaining good health.

Key words: *Coronavirus; College students; Nutrition; Micronutrients; Daily activities; Online survey; Questionnaire.*

INTRODUCTION

The world is facing the COVID-19 pandemic caused by the SARS-CoV-2 virus (referred to as the COVID-19 virus). Many countries are following the advice given by the WHO [1] regarding the introduction of physical distancing measures. People in many countries are under quarantine, in order to reduce the spread of the virus.

COVID-19 is a respiratory illness and the primary transmission route is through contact between people and through direct contact with respiratory droplets generated when an infected person coughs or sneezes. Unlike foodborne gastrointestinal viruses, such as norovirus and hepatitis A, there is currently no evidence that food or food packaging is a likely source or route of transmission of the COVID-19 virus [1-4]. Besides that, scientists and authorities across the world are monitoring the spread of the virus and there have not been any reports of transmission through food.

Currently, there is no registered treatment or vaccine for the disease. In the absence of a specific treatment for this novel virus, there is an urgent need to find an alternative solution to prevent and control the replication and spread of the virus. Studies have been published proposing a specific diet as therapeutic options available for the treatment of this novel coronavirus [5]. WHO/Europe [6] has published a new guide on how to eat healthily during the COVID-19 self-quarantine. It contains valuable information about nutrition to help keep the immune system strong.

Good nutrition and physical activity are major determinants of health and disease [7]. Nevertheless, a longer stay in isolation and quarantine can prevent a good and healthy diet and physical activity. The student population is usually very socially active. Students usually spend a large part of the day outside the home. Therefore, isolation and quarantine can have a significant effect on changing the daily activity of this population as well as their diet.

From March 16th to May 6th 2020, a state of emergency was in force in Serbia due to the coronavirus pandemic and all residents were obliged to be in quarantine. During that period, among other things, the faculties and dormitories were closed and students were at their homes. Given the importance of nutrition and great changes in daily activities, the aim of this study was to examine nutrition and behavior of students during COVID-19 quarantine, with special reference to certain microelements that are necessary for body's defense against the new coronavirus.

PARTICIPANTS AND METHODS

This research was conducted using a questionnaire. Participants answered an online survey questionnaire. Data collection was completed during the May 2020. Students gave written consent to participate in the survey and data processing. This questionnaire is in line with the General Regulation on Data Protection of the European Union. The collected data were used exclusively for scientific purposes. This research is a part of a broader study titled "Attitudes and behavior of students in relation to food and nutrition" conducted among students of the Faculty of Agriculture, University of Belgrade, on the subject of "Food Biochemistry".

The study sample included 114 students of the second year of bachelor studies at the Institute of Food Technology and Biochemistry, Faculty of Agriculture, University of Belgrade, between the ages of 20 and 23 (the average age was 21.78±1.3) and all were of Serbian nationality. The sample was composed of 90 females (78.0%) and 24 males (21.0%), **Table 1**. The surveyed students were selected randomly; all students had an equal chance of being selected for the sample.

Descriptive statistics methods were used to process and analyze the data. Version 8.0 software (StatSoft Co., Tulsa, Oklahoma, USA) was used for statistical processing of the results.

RESULTS AND DISCUSSION

Nutritional habits and behavior in quarantine

Current reports in the world about patients with COVID-19 show that this virus "attacks" all population. However, the latest clinical research suggests that COVID-19 is associated with negative outcomes in older and comorbid patients [8]. These characteristics are not specific to the student population. None of the participants were infected with COVID-19, even if seven students reported their chronic non-communicable disease. Such a good result can be a consequence of the correct behavior in quarantine and proper diet. The emerging studies on patients with COVID-19 indirectly highlight the relevance of nutrition. Namely, these new

Table 1. Participants' socio-demographic characteristics, individual habits and health status (N=114).

Characteristics	**n**	**%**
Gender		
male	24	21.0
female	90	78.0
Age groups (average age – 21.78±1.3)		
21–23	114	100.0
Class status		
2nd year	114	100.0
Nationality		
Serbian	114	100.0
Physical activity		
yes, every day	17	14.9
yes, 1–2 per week	30	26.3
yes 3–4 per week	32	28.1
no	35	30.7
Activities in quarantine conditions		
studying	84	73.7
hobby	23	20.2
I'm bored	7	6.1
Have you been sick of COVID-19?		
yes	–	–
no	114	100.0
Have someone from your family been sick of COVID-19?		
yes	3	2.6
no	111	97.4
Are you under stress due to constant information about the pandemic?		
yes	39	34.2
no	75	65.8
Are you under stress for fear of illness?		
yes	26	22.8
no	88	77.2
Do you think quarantine has a negative impact on your mental health?		
yes	35	30.7
no	79	69.3
Do you suffer from a chronic non-communicable disease?		
yes	7	6.1
no	107	93.9
Do you sleep well at night?		
yes	88	77.2
no	26	22.8
Have you spent any time in the Sun?		
yes	100	87.7
no	14	12.3

studies indicate that the presence of comorbid conditions with impaired nutritional status, a high body mass index score and sarcopenic obesity may have an impact on the occurrence and outcome of this disease [9,10] Peng et al. [10] pointed out that higher BMI is more common in critical patients and non-survivors, so that as many as 88.24% of non-survivors had a BMI>25 kg/m^2. According to the WHO [11] we can classify the weight of adults according to BMI: underweight (BMI <18.5 kg/m^2), normal weight (BMI=18.5-24.9 kg/m^2), overweight (BMI=25-29.9 kg/m^2), and obese (BMI>30 kg/m^2). Most of the surveyed students were not at this risk, although 23% of students reported a BMI>25 kg/m^2 and 58.8% of the students had a BMI that fits in "normal weight" (**Table 2**). However, even over 80% (**Table 2)** of the respondents think that they are not obese, which is contrary to the reported BMI. This indicates that the surveyed students need education in this regard.

New guidelines for preventive defense against this virus prescribe the intake of a sufficient amount of energy [12]. Larger proportion of surveyed students (64.0%) stated that they did take higher amount of energy than needed daily (**Table 3**). Need for higher energy intake is in line with a significant number of students who were physically active (69.3%; **Table 1**) at the time of quarantine. Many studies promote physical activity as a significant potential for reducing the severity of some diseases and improving the quality of life in adults [13].

Of the surveyed students 73.7% spent most of their time completing college assignments and studied, while 20.2% of the surveyed students spent a large part of their time in activities related to their hobbies, and 6.1% of the surveyed students were bored (**Table 1**). The appearance of boredom was expected, since quarantine is associated with the interruption of work routine. But, boredom has been associated with a greater energy intake, as well as the consumption of higher quantities of proteins, fats and carbohydrates, as well as with increased desire to snack and consume less healthy foods [14]. Although most students (61.5%) had regular three meals a day (breakfast (81.6%), lunch (90.4%), and dinner (72.8%), there were respondents who also had a snack (more than three meals a day; 19.3%), **Table 2**. However, since most students (70.2%) did not gain weight during quarantine (**Table 2**), we can assume that their snacks mostly were not high calorie.

Muscogiuri and associates [15] stated that during quarantine continuously hearing or reading about the pandemic without a break can be stressful. In this regard Yilmaz et al. [16] concluded that the stress pushes people toward overeating, mostly looking for sugary "comfort foods". Although most of the surveyed students (65.8%) did not feel stress due to the pandemic,

Table 2. Characteristics of nutritional behavior in quarantine (N=14).

Questions	Answers	
	n	%
Do you have a constant need for food?		
yes	42	36.8
no	72	63.2
Do you eat a much larger amount of food than usual?		
yes	37	32.5
no	77	67.5
Have you gained weight?		
yes	34	29.8
no	80	70.2
Do you think you are obese?		
yes	20	17.5
no	94	82.5
What is your body mass index?		
<18.5	20	17.6
18.5–24.9	67	58.8
25–29.9	17	14.9
30–34.9	7	6.1
35–39.9	3	2.6
≥40	–	–
How many meals do you have per day?		
1	1	0.9
2	22	19.3
3	69	60.5
more than 3	22	19.3
Do you have a regular breakfast?		
yes	93	81.6
no	21	18.4
Do you have a regular lunch?		
yes	103	90.4
no	11	9.6
Do you have a regular dinner?		
yes	83	72.8
no	31	27.2
Are you ordering food to be delivered to your home?		
yes	11	9.6
no	103	90.4
Do you miss eating in restaurants?		
yes	27	23.7
no	87	76.3
Have you succumbed to excessive buying food?		
yes	9	7.9
no	105	92.1

Table 3. Energy and nutritional value of food.

Questions	Answers	
	n	%
N=114		
Are you careful about the nutritional and energy value of food?		
yes	41	36.0
no, I eat what I like	51	44.7
no, I eat according to my capabilities	22	19.3
Do you take more energy per day than you need?		
yes	73	64.0
no	41	36.0
Do the foods you consume contain carbohydrates?		
yes	99	86.8
no	15	13.2
Do the foods you consume contain fat?		
yes	83	72.8
no	31	27.2
Do the foods you eat contain protein?		
yes	114	100.0
no	–	–
Do the foods you consume contain antioxidants?		
yes	93	81.6
no	21	18.4
Do the foods you consume contain vitamins?		
yes	112	98.2
no	2	1.8
Do the foods you consume contain minerals?		
yes	109	95.6
no	5	4.4
N=83		
***Do you have dinner late in the evening?**		
yes	31	37.4
no	52	62.6
***Is your dinner rich in carbohydrates?**		
yes	42	50.6
no	41	49.4

a significant number of respondents were stressed (34.2%), and even believe that quarantine had a negative effect on their mental health (30.7%; **Table 1**). Besides that, Rodríguez-Martín and Meule [17] defined this desire to consume a certain type of food as "food craving" and these foods usually have high palatability and are energy dense and have high fat and/or sugar content. "Food craving" is a multidimensional phenomenon including behavioral (e.g., seeking and consuming food), cognitive (e.g., thinking about food), emotional (e.g., desire to eat), and physiological (e.g., salivation) processes [18,19]. Although most of the surveyed students (63.2%) did not feel constant need for food, a significant number of respondents (36.8%) felt constant need for food and in addition, a significant number of respondents (32.5%) ate much larger amounts of food than usual (**Table 2**).

Quarantine-related stress has also resulted in sleep disorders in a significant number of students (22.8%; **Table 1**). This in turn can further exacerbate stress and increase food intake perpetuating a "vicious circle" that can endanger health. That is why it is important to consume food that stimulates the secretion of serotonin and melatonin. However, there is also a trap here that can lead to a "vicious circle". Namely, foods rich in carbohydrates stimulate the production of serotonin, which in turn has a positive effect on mood. Almost all of the students surveyed (86.8%) consumed foods rich in carbohydrates. In addition, of those students who answered that they had a regular dinner (83 students) 50.6% of students ate carbohydrates and 37.4% of them had dinner in the late evening (after 10 pm; **Table 3**). This unhealthy nutritional habit could increase the risk of developing obesity, hypertension, diabetes, and lung disease that have been demonstrated to increase the risk for more serious complications of COVID-19 [20]. It is much better to consume other foods that contain melatonin and/or serotonin such as: root and green-leafy vegetables, fruits such as bananas and cherries and nuts (walnuts, hazelnuts, cashew, and almonds). These foods also contain tryptophan, which is a precursor of serotonin and melatonin [21]. In addition to these foods, protein foods such as milk and milk products, eggs (white), meat, fish are the main sources of tryptophan [21]. Tryptophan is involved in the regulation of satiety and caloric intake via serotonin that mainly lowers carbohydrate and fat intake. Moreover, tryptophan is a sleep-inducing amino acid [22]. A review of individual groups of foods consumed by students indicated a presence in the diet of foods that can be sources of tryptophan (**Table 4**).

Macronutrients and micronutrients

Usually during quarantine, the intake of macronutrients is increased and the intake of micronutrients is reduced which can lead to obesity [15,23]. All macronutrients (fats, proteins, carbohydrates) were present in the daily diet of the students. To questions related to the composition of foods consumed during quarantine, students answered that the foods contained:

Table 4. Foods consumed by the surveyed students (N=114).

Foods*	yes		no	
	n	%	n	%
spinach, chard, greens, lettuce ...	108	94.7	6	5.3
potatoes	109	95.6	5	4.4
beets, celery, parsley …	76	66.7	38	33.3
carrots	101	88.6	13	11.4
peas, green beans, beans …	109	95.6	5	4.4
soybeans products	22	19.3	92	80.7
onion, garlic, leek…	101	88.6	13	11.4
cauliflower, broccoli, artichoke…	50	43.9	64	56.1
asparagus, kohlrabi…	15	13.2	99	86.8
mushrooms	85	74.6	29	25.4
wheat germ	51	44.7	63	55.3
sunflower seeds	67	58.8	47	41.2
pumpkin seeds	46	40.4	68	59.6
sesame seeds	79	69.3	35	30.7
maize	77	67.5	37	32.5
whole-flour	22	19.3	92	80.7
walnuts, almonds, hazelnuts ...	71	62.3	43	37.7
bananas, pineapples, dates ...	92	80.7	22	19.3
citrus fruits (lemon, oranges ...)	101	88.6	13	11.4
raspberries, blackberries, strawberries, blueberries ...	97	85.1	17	14.9
apples, pears ...	101	88.6	13	11.4
plum, peach ...	67	58.8	47	41.2
milk and cheese	110	96.5	4	3.5
milk yoghurt (from milk of animal origin)	104	91.2	10	8.8
fish	101	88.6	13	11.4
liver	23	20.2	91	79.8
eggs	114	100.0	–	–
poultry meat	112	98.2	2	1.8
„red meat"	93	81.6	21	18.4

* Foods can be fresh, dried or frozen.

carbohydrates (86.8%), fats (72.8%), and proteins (100.0%). Although 64.0% of respondents did not care about the energy and nutritional values of food (44.7% ate the food they liked, while 19.3% of respondents ate according to their abilities, regardless of nutritional and energy value of foods; **Table 3**), the diet of the examined students was obviously well balanced, considering that no student suffered from COVID-19. Students had well-balanced meals that had a beneficial effect on their immune responses.

Most of the students ingested minerals (95.6%), antioxidants (81.6%) and vitamins (98.2%, **Table 3**) mostly through meals, not through dietary supplements. Several studies reported that fruits and vegetables supplying micronutrients can boost immune function [24,25]. Micronutrients important for a good immune response are antioxidants, vitamin A and β-carotene, vitamin C and vitamin D, as well as zinc and selenium. Anti-oxidants (such as: vitamin E, selenium, zinc) increase the number of T-cell subsets, enhance lymphocyte response [26]. Deficiency in selenium induces not only impairment of host immune system, but also rapid mutation of benign variants of RNA viruses to virulence [27]. Zhang and Liu [5] pointed out that selenium supplementation could be an effective choice for the treatment of this novel virus, COVID-19. A relatively small number of students (15 students, 13.2% of respondents) used dietary supplements as addition to their diet. But a large number of students consumed foods that could be a source of selenium (**Table 4**),

such as: red meat, fish, milk, eggs, whole grains, onions, tomatoes, nuts and different seeds [27]. Selenium often exhibits antioxidant activity (prevents the formation of free radicals and prevents oxidative damage to cells and tissues) in synergy with vitamin E [5]. Vitamin E is found primarily in plant products, the richest sources being plant oils (soybean, sunflower, corn, wheat germ, and walnut), also green plants tend to contain vitamin E (spinach and broccoli) as well as, nuts and seeds [5,28]. Except soy-food, students mostly consumed foods that could be a source of vitamin E (**Table 4**).

Vitamin C also supports immune functions and protects against infection caused by the SARS coronavirus [29]. Further, vitamin C may function as a weak antihistamine agent to provide relief from flu-like symptoms such as sneezing, a runny or blocked-up nose, and swollen sinuses [30]. Sources of vitamins C include: peppers, broccoli, cabbage, oranges, strawberries, mangoes, lemons, and other fruits and vegetables, and organ meats (e.g., liver and kidney) [5,28]. Apart from mango, which is not a traditional fruit in the Balkans, and liver, which was consumed by a relatively small number of students, other foods that can be sources of vitamin C were consumed by a large number of students (**Table 4**). In addition, all students who used dietary supplements used vitamin C as well as zinc (**Table 5**).

Zinc is another essential trace element that is crucial for the maintenance of immune function [31], in addition, it has been reported that zinc at low concentrations inhibits the replication of SARS coronavirus [32]. Zhang and Liu [5] pointed out that zinc supplement may have effect not only on COVID-19-related symptom like diarrhea and lower respiratory tract infection, but also on COVID-19 itself. The most common food to get zinc is represented from: nuts, pumpkin seeds, sesame seeds, beans, red meat and poultry [5]. The results of this study showed that a significant number of students consumed foods that could be a source of zinc (**Table 4**).

Ebadi and Montano-Loza [33] pointed out that potential immunomodulators may help alleviate severity and improve the outcomes of these diseases. Vitamin D has a wide spectrum of anti-inflammatory, antifibrotic, immunomodulatory, and antioxidant actions. The same authors have found that severe vitamin D deficiency is associated with disease progression and increased mortality in patients [33]. Epidemiological studies have reported that vitamin D deficiency is associated with viral respiratory tract infections and acute pneumonia [34]. Zhang and Liu [5] pointed out that vitamin D could work as another therapeutic option for the treatment of this novel virus. Namely, vitamin D reduces the risk of developing several chronic diseases such as: cardiovascular disease and hypertension, diabetes mellitus, metabolic bone diseases, and cancers. Adequate vitamin D status protects respiratory tract, therefore reducing the risk of pneumonia [15, 28,35,]. Vitamin D, the "sunshine vitamin," is actually a hormone produced from sterols in the body by the photolytic action of ultraviolet light on the skin [28]. However, quarantine could be associated to less sun-exposure, and reduced production of vitamin D. Nonetheless, most of the surveyed students spent time in the sun (87.7%; **Table 1**). Still, as the appearance of COVID-19 and quarantine covered mostly the winter months, and consequently the sun exposure was limited, it was encouraged to get more vitamin D from diet. Vitamin D, as either ergocalciferol (vitamin D2) or cholecalciferol (vitamin D3), is rather sparsely represented in nature and few foods are rich in vitamin D. The richest natural sources are fish liver, and oils (cod, tuna and mackerel oils are particularly rich sources of vitamin D3). However, these foods are not usually used every day in Serbia, and in addition, their price is very high. Therefore, the population uses fish, liver, egg yolk and foods with added vitamin D (e.g., milk, yogurt) as sources of vitamin D [28]. A relatively small number of students consumed liver, but a large number of students used fish, eggs, milk and dairy products (**Table 4**). Vitamin D can also be obtained from nutritional supplements, but few students have used vitamin D in the form of capsules and tablets (**Table 5**). Perhaps students should be encouraged (especially in the winter months) to use vitamin D in the form of dietary supplements.

The B vitamin group is very important in viral infections for the host immune response. Zhang and Liu [5] pointed out those B vitamins could be chosen as a basic option for the treatment of COVID-19. Given that these vitamins are found in a large number of foods of plant and animal origin, it is considered that

Table 5. Use of dietary supplements.

Questions	Answers	
	n	%
Do you use dietary supplements? (N=114)		
yes	15	13.2
no	99	86.8
*I take from supplements: (N=15)		
vit D	8	55.3
vit C	12	100.0
vit E	2	13.3
vit A (or β–carotene)	6	40.0
essential fatty acids	1	6.7
zinc	12	100.0
selenium	5	33.3

* This question was only applied to students who answered that they take supplements (most of them take several different supplements, not just one) (N=15).

proper nutrition can be ingested in sufficient quantities. Foods containing B vitamins include: red meat, liver (beef), fish, eggs, milk and dairy products, rice, whole-flour, celery, cabbage, beans, asparagus, broccoli, spinach, carrots, cauliflower, potatoes, tomatoes, cucumber, corn, soy, oranges, apples, grapefruit, pineapple, peaches, strawberries, plums, and nuts. The exception is vitamin B12, whose source is only in foods of animal origin that were previously listed [28]. Students consumed most of these foods (**Table 4**), so it can be assumed that they ingested a sufficient amount of B vitamins.

CONCLUSIONS

A large number of students did not belong to the risk group for COVID-19. Only a few students suffer from chronic diseases and a small number of students are obese. A significant number of surveyed students were careful about the nutritional and energy value of food. The diet of the examined students was well balanced, considering that no student suffered from COVID-19. The students had well-balanced meals that had a beneficial effect on their immune response. The students had all the macronutrients in their diet during COVID-19 quarantine. Most of the students ingested micronutrients mostly through meals. A relatively small number of students ingested minerals, antioxidants, and vitamins through dietary supplements. Students should be encouraged to use vitamin D in the form of dietary supplements, especially in the winter months. Generally, this research may help for a better understanding of the importance of a proper and balanced diet for maintaining good health. In addition, it can have an educational significance on the student population about proper nutrition as well as about the need to use dietary supplements during pandemics.

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Ishrana studenata tokom karantina zbog COVID-19

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Kratak sadržaj

Svetska zdravstvena organizacija je COVID-19 proglasila pandemijom i veliki broj ljudi je bio ili je u karantinu. Tokom karantina, kontinuirano slušanje ili čitanje o pandemiji može imati efekte na različite aspekte života ljudi. Jedan od ovih vrlo značajnih efekata je na ishranu ljudi. Cilj ove studije bio je da sumira iskustva studentske populacije o ponašanju i kvalitetu ishrane tokom karantina COVID-19, sa posebnim osvrtom na određene mikroelemente koji su neophodni za imunološki odgovor. Studenti su pozvani da urade online anketu tokom maja 2020. Nijedan od učesnika nije bio zaražen sa COVID-19. Od ispitanih studenata 34.2% je bilo pod stresom zbog stalnih informacija o pandemiji. Značajan broj studenazta je imao dobre prehrambene navike. Većina ispitanika nije osećala stalnu potrebu za hranom (63.2%), niti su konzumirali veće količine hrane nego obično (67.5%). Studenti (36.0%) su bili pažljivi u pogledu nutritivne i energetske vrednosti hrane. Većina ispitivanih studenata (86.8%) je unosila mikroelemente uglavnom hranom. Studenti su imali dobro uravnotežen obrok koji je blagotvorno uticao na njihov imunološki odgovor. Veoma mali broj studenata (13.2%) je koristio dijetetske suplemente. Generalno, ovo istraživanje može pomoći boljem razumevanju važnosti pravilne i uravnotežene ishrane i upotrebe dijetetskih suplemenata radi održavanja dobrog zdravlja.

Ključne reči: *Koronavirus; Studenti; Ishrana; Mikroelementi; Dnevne aktivnosti; Online anketni upitnik.*

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In vitro antioxidant activity evaluation of rosmaric acid and honey based supplement

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Abstract

Disrupted balance between free radicals and antioxidants leads to a pathophysiological state of oxidative stress, which is the underlying cause of many diseases. Since some synthetic antioxidants show some potentially adverse effects, many studies aim to find natural antioxidants. Secondary plant metabolites, such as polyphenols, phenolic acids, which include rosmarinic acid, terpenes and terpenoids have antioxidative properties and can be found in different hydrosols and honey. The aim of this study is to evaluate the in vitro antioxidative activity of the supplement based on hydrosol mixture, rosmarinic acid and honey. A series of diluted (100%, 50%, and 25%) supplements were added to the serum pool, collected from healthy donors. A second series of samples was prepared with the same amounts of supplement and addition of tert-Butyl hydroperoxide (TBH) as a prooxidant. Alongside these samples, sera samples with Trolox (hydrophilic vitamin E analog) and Trolox with TBH were prepared. All these samples were tested for the following oxidative stress parameters: total antioxidative status (TAS), paraoxonase-1 (PON-1), total sulfhydryl groups content (SHG), total oxidative status (TOS), prooxidative-antioxidative balance (PAB) and advanced oxidation protein products (AOPP). TAS/TOS ratio was calculated as a quantitative measurement of antioxidative and oxidative substances ratio in the serum. Antioxidative parameters TAS, SHG, TAS/TOS ratio and prooxidant/antioxidant balance parameter PAB, were not significantly different for the supplemened samples with and without the addition of TBH, which indicates that the supplement keeps its antioxidative properties, despite the addition of TBH. The supplement showed significant antioxidative properties as a result of the synergistic effect of rosmarinic acid, terpenes, and terpenoids from hydrosols and polyphenols and other antioxidant substances from honey.

Key words: *Oxidative stress; Antioxidants; Hydrosol; Terpenes; Polyphenols; Rosmarinic acid; Honey.*

INTRODUCTION

Free radicals represent atoms, ions or molecules which have one or more unpaired electrons in their outer shell, making them very reactive towards the other molecules. Antioxidants are molecules which have the ability to neutralize free radicals action. In the order for the normal physiological processes to take place, the balance between free radicals and antioxidants is necessary. Disrupting that balance in any way, could lead to pathophysiological state called oxidative stress. Due to the free radicals ability to damage lipids, proteins and DNA, it is understandable that oxidative stress may be a cause of many diseases. For that reason, the additional intake of antioxidants may be helpful in order to keep balance between free radicals and antioxidants. Since some of the synthetic antioxidants showed some potentially adverse effects, there are more studies aiming to find natural antioxidants, which should have less adverse effects and more efficiency [1,2].

Medical plants of the family *Lamiaceae* produce wide range of secondary metabolites, including antioxidants, such as ascorbic acid, tocopherol, carotenoids, terpenes and polyphenols. Polyphenols are large group of phytochemical compounds and their antioxidant activity depends on their structure and aromatic rings substituents. Flavonoids, tannins and phenolic acids, such as rosmarinic acid, are polyphenols with high antioxidative activity [3]. Some studies suggested that diets rich in plant polyphenols can offer some protection against development of cancer, cardiovascular diseases, diabetes, osteoporosis and

neurodegenerative diseases [4]. Over 8000 polyphenolic compounds have been found in different plant species, of which the largest group with the most bioactive potential are flavonoids [5]. The mechanisms involved in the antioxidant capacity of polyphenols include suppression of reactive oxygen species (ROS) formation by either inhibition of enzymes involved in their production, scavenging of ROS, metal ion chelation ability, or upregulation or protection of antioxidant defenses. Some polyphenols may react in plasma membrane with nonpolar compounds present in the hydrophobic inner membrane layer. In this way, they can affect oxidation rate of lipids or proteins and may prevent access of oxidants and protect the structure and function of the membrane [6].

Rosmarinic acid (RA) was, for the first time, isolated from rosemary leaves (*Rosmarinus Officinalis*), and it has been later isolated from other *Lamiaceae* and *Boraginaceae* family plants. Despite the fact that RA has been isolated from various plants, rosemary is still the biggest RA source. Rosmarinic acid is caffeic acid and 3,4-dihydroxyphenylacetic acid ester. Caffeic acid part of RA originates from phenylalanine, while 3,4-dihydroxyphenylacetic acid part of RA originates from tyrosine [7–9]. Antioxidative effect of phenolic acids, including rosmarinic acid, depends on their structure, substituent and its position on aromatic ring and side chain structure. Greater number of hydroxyl and methoxy groups, especially presence of o-hydroxyl groups on phenolic group, increases antioxidative activity [10]. RA works as a ROS "catcher" by hydrogen donating. More precisely, o-hydroxyl groups in A and B rings are electron donors. This leads to forming of semiquinone and quinone structures, which stabilizes newly formed free radical structure [11]. Furthermore, RA inhibits enzyme xanthine-oxidase (it catalyzes hypoxanthine to xanthine reaction, followed by xanthine to uric acid reaction, while creating H_2O_2), which is one of endogenic sources of free radicals in some pathophysiological states like ischemia or tissue damage. RA also reduces Mo(VI) to Mo(V), reducing metal induced free radical forming [11,12]. RA can infiltrate into the inner, nonpolar layer of plasma membrane, preventing lipid peroxidation by intercepting intramembrane radicals and increasing membrane flow, reorganizing lipid chains and making free radical propagation harder [13]. In addition, RA increases production of enzymes that participate in primary antioxidative protection, such as superoxide-dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GPx) [14]. RA prevents DNA damage with its antioxidative properties, while it also induces damaged DNA reparation by mediating intracellular mechanism responsible for DNA reparation [15].

Beside polyphenols, terpenes also belong to a significant group of plants' secondary metabolites. Their antioxidative activity is based on ROS "catching" and effect on endogenous antioxidants. Terpenes also show anti-inflammatory, antiallergic, anticoagulative, antitumor, as well as, sedative and analgesic effect [3,16]. Terpenes activity depends on their concentration. Low concentrations of terpenes show antioxidative effect, but high concentrations show prooxidative effect [17].

Alongside plants, polyphenolic compounds can be found in honey, which gives honey a significant antioxidative properties, with its' primary antibacterial activity. Antioxidative properties of honey are based on flavonoids, phenolic acids, vitamins C and E, enzymes (catalase and peroxidase) and trace elements. Antibacterial and antioxidative properties of honey are connected, they effect on each other and together they give special therapeutic characteristics [18].

Hydrolats (hydrosols, flower waters) represent a water phase that forms during steam or water distillation of aromatic plants in essential oil production process. Hydrolat composition is very different from the essential oil of the same plant, because of the water solubility of components, which gives different activity to oils and hydrolats [19]. Main components of hydrolats are, mostly, terpenes and terpenoids, which give hydrolats their antioxidative, as well as antibacterial, antiviral, and other properties of different hydrolats [20].

The aim of this study was to evaluate the *in vitro* antioxidative activity of the supplement based on hydrosol mixture, rosmarinic acid and honey in biological material. The supplement is defined as functional beverage, intended for human diet. This model enables us to predict supplement's activity in blood after the predicted resorption.

MATERIAL AND METHODS

Study design

Med(i)ra supplement (ROSA VITA d.o.o, Pula, Croatia) is a herbal complex based on hydrosol mixture, rosmarinic acid (75 mg RA per 10 mL of drink) and honey. Rosemary (*Rosmarinus officinalis*) hydrosol, lavender (*Lavandula angustifolia*) hydrosol, fennel (*Foeniculum vulgare*) hydrosol and cade juniper (*Juniperus oxycedrus*) hydrosol were used for the mixture. Honey, used in this supplement, originates from aromatic plant fields, which are also used for hydrosol production.

A series of diluted (100%, 50%, and 25%) supplement (25 µL) was added to the serum pool (450 µL), collected from healthy donors. Since there wasn't any significant difference in results between different dilutions of a supplement, we pooled all results from series of dilutions into one group. A second series of samples was prepared with the same supplement amounts and tert-Butyl hydroperoxide (TBH) added as a prooxidant. Alongside these samples, sera samples with Trolox (hydrophilic vitamin E analog) and Trolox with TBH were prepared. All these samples were tested for the fol-

lowing oxidative stress parameters: total antioxidative status (TAS), paraoxonase-1 (PON-1), total sulfhydryl groups content (SHG), total oxidative status (TOS), prooxidative-antioxidative balance (PAB) and advanced oxidation protein products (AOPP). TAS/TOS ratio was calculated as a quantitative measurement of antioxidative and oxidative substances ratio in the serum.

Laboratory analysis

Each of the hydrosols used in herbal mixture was analyzed with gas chromatography – mass spectrometry (GC-MS) on HP-5MS capilary column. *(Faculty of Chemistry & Technology, Split, Croatia)*.

Total antioxidative status (TAS) was determined by a colorimetric test using stable 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) cation as a chromogen. The reaction was performed on the *Ilab 300+ Instrumentation Laboratory, (Milan, Italy)*. Paraoxonase-1 (PON-1) activity was measured in a kinetic reaction based on interaction of PON1 enzyme, from the sample, with the paraoxone substrate. The Ellmann method was performed in order to determine total sulfhydryl groups content (SHG). Total oxidative status (TOS) was measured spectrophotometrically after colorimetric reaction in which the oxidants presented in the sample oxidize the ferro-ortho-dianiside complex to the ferric ion. Advanced oxidation protein products (AOPP) was measured after the addition of glacial acetic acid to a diluted sample with phosphate buffer (pH 7.4) and a potassium iodide solution. All PON-1, SHG, TOS and AOPP analyses were performed on *Ilab 300+ Instrumentation Laboratory, (Milan, Italy)*. Prooxidative-antioxidative balance (PAB) test is based on colorimetric reaction of chromogen 3,3',5,5'-tetramethylbenzidine (TMB) with H_2O_2 and antioxidants (uric acid) at the same time, since they are in the same serum sample. The calibration curve is formed and used for the results calculation. [21]

Statistical analysis

All data are presented as median and interquartile range (25th and 75th percentiles). Mann-Whitney U test was used to calculate p-values between sample groups for each of the parameters. A p-value of less than 0.05 was considered statistically significant. All data were analyzed using IBM® SPSS® ver. 26.0 (IBM, Armonk, USA) software.

RESULTS

Table 1 represents some of the most significant compounds of hydrosols used in the supplement.

Table 1. The most significant compounds of hydrosols used in the Med(i)ra supplement (represented as peak area %).

	Juniperus oxycedrus	*Lavandula angustifolia*	*Rosmarinus officinalis*	*Foeniculum vulgare*
Compound	Peak area %			
1. 1,8-Cineole	0.3	0.2	6.4	5.3
2. Fenchone	0.4	–	–	40.0
3. Linalool	1.4	19.6	2.4	4.6
4. *cis*-Linalool oxide	–	3.8	0.1	–
5. *trans*-Linalool oxide	–	3.5	–	–
6. Borneol	1.9	5.8	10.9	–
7. Terpinene-4-ol	2.5	17.0	3.4	3.2
8. *p*-Cymen-8-ol	3.4	2.2	0.3	0.3
9. α-Terpineol	7.6	13.6	8.3	2.9
10. Verbenone	3.4	0.4	41.8	–
11. *trans*-Carveol	3.6	0.2	0.2	0.2
12. *cis*-Carveol	1.4	–	–	–
13. Carvacrol	2.1	–	–	–
14. Methyleugenol	4.3	0.6	1.2	2.3
15. 4-Methyl-2,6-bis(1,1-dimethylethyl)- phenol	14.8	1.9	2.0	8.8
16. (*Z*)-9-octadecen-1-ol	2.8	0.3	0.3	7.2
17. Camphor	–	0.7	7.6	1.7
18. Methyl Chavicol (Estragole)	–	–	–	4.4
19. Geraniol	–	–	0.3	0.3
20. Eugenol	1.3	0.2	0.6	0.8
21. 3,7-Dimethyloct-1-en-3,7-diol	–	4.0	0.2	–
21. Coumarin	–	3.1	–	0.2
Total identified	76.5 %	90.2 %	91.3 %	89.7 %

The main compounds of cade juniper (*Juniperus oxycedrus*) hydrosol are 4-methyl-2,6-bis(1,1-dimethylethyl)- phenol (14.8%) (used in BHT production) and α-terpineol (7.6%). Linalool (19.6%), terpenine-4-ol (17.0%) and α-terpineol (13.6%) dominate in lavender (*Lavandula angustifolia*) hydrosol, while the main compounds of rosemary (*Rosmarinus officinalis*) hydrosol are verbenone (41.8%), borneol (10.9%) and α-terpineol (8.3%). Fenchone (40%) is the main compound in fennel (*Foeniculum vulgare)* hydrosol. 1,8-cineol is one of the terpenes with significant antioxidant activity and can be found in all hydrosols in varies amounts (0.2-6.4%).

The concentrations of oxidative stress parameters in samples without TBH addition are shown in **Table 2**.

Table 2. The concentrations of oxidative stress parameters in samples without added TBH.

	Medi(r)a + serum pool (*c*=25%,50%, 100%)	Serum	Trolox + serum pool
TAS (μmol/L)	1638 (1598-1657)	662 [a] (628-696)	810 [a] (752-868)
TOS (μmol/L)	125 (123-130)	123 (111-136)	113 [a] (110-116)
PAB (U/L)	3.23 (2.77-4.23)	97.8 [a] (94.6-101)	84.6 [b] (81.6-87.5)
AOPP (μmol/L)	90.6 (90.4-90.6)	118 [a] (112-125)	118 [a] (114-121)
SHG (mmol/l)	1.17 (0.84-1.73)	0.55 [a] (0.53-0.58)	0.51 [a] (0.50-0.51)
PON1 (U/L)	208 (198-216)	170 [a] (164-176)	184 (182-185)

a - Med(i)ra vs serum; Med(i)ra vs Trolox; b – serum vs Trolox; a, b, p<0,05 (compared using non-parametric Mann-Whitney U test)

Antioxidative parameters TAS and SHG were significantly higher ($p<0.05$), while PAB and oxidative paremeter AOPP were significantly lower ($p<0.05$) in all Med(i)ra samples without added TBH compared to serum and Trolox samples without TBH addition. PON1 parameter was significantly higher in Med(i)ra samples than in serum samples, while TOS was the only parameter that showed significantly lower value in Trolox samples compared to Med(i)ra samples ($p<0.05$).

The concentrations of oxidative stress parameters in samples with TBH addition are shown in **Table 3**.

After TBH has been added to all samples, antioxidative parameters TAS and SHG were higher ($p<0.05$), while PAB was significantly lower ($p<0.05$) in Med(i)ra samples compared to serum and Trolox samples. The other oxidative stress parameters, TOS, AOPP and PON1, didn't show any significant difference in samples after TBH addition. Also, it has been noticed that there wasn't significant difference between oxidative stress parameters measured in Trolox and serum samples.

Table 3. The concentrations of oxidative stress parameters in samples with TBH addition.

	Medi(r)a (*c*=25%,50%,100%) with TBH	Serum with TBH	Trolox with TBH
TAS (μmol/L)	1605 (1584-1642)	734 [a] (712-756)	752 [a] (733-771)
TOS (μmol/L)	127 (125-128)	129 (127-130)	126 (124-128)
PAB (U/L)	2.30 (1.97-3.10)	113.10 [a] (112-114)	96.0 [a] (95.4-96.6)
AOPP (μmol/L)	109 (91.0-129)	122 (121-123)	120 (115-124)
SHG (mmol/l)	0.77 (0.64-0.98)	0.47 [a] (0.46-0.48)	0.48 [a] (0.44-0.52)
PON1 (U/L)	195 (169-213)	217 (188-247)	196 (194-199)

a - Med(i)ra with TBH vs serum with TBH; Med(i)ra with TBH vs Trolox with TBH; b – serum with TBH vs Trolox with TBH; a, b - p<0,05 (compared using non-parametric Mann-Whitney U test)

Table 4. Med(i)ra with added TBH vs Trolox without added TBH.

	Medi(r)a (*c*=25%,50%,100%) with TBH	Trolox	***p***
TAS (μmol/L)	1605 (1584-1642)	810 (752-868)	***<0,05***
TOS (μmol/L)	127 (125-128)	113 (110-116)	***<0,05***
PAB (U/L)	2.30 (1.97-3.10)	84.6 (81.6-87.5)	***<0,05***
AOPP (μmol/L)	109 (90.8-129)	118 (114-121)	*1,00*
SHG (mmol/l)	0.77 (0.64-0.98)	0.51 (0.50-0.51)	***<0,05***
PON1 (U/L)	195 (169-213)	184 (182-185)	*0,505*

When the concentration values of parameters measured in Med(i)ra samples with added TBH were com-

pared to the concentration values measured in Trolox samples without added TBH, it has been noticed that despite TBH addition, Med(i)ra showed a more favorable redox status comparing to serum sample. Data are showed in **Table 4**.

All oxidative stress parameters, except PON1 and AOPP, measured in Med(i)ra samples with added TBH and Trolox samples without added TBH showed significant difference with $p<0.05$. TAS and SHG were significantly higher and PAB was significantly lower in Med(i)ra samples despite TBH addition, while only TOS was significantly lower in Trolox samples without added TBH.

In order to show more precise relation between antioxidative capacity and oxidative stress in all samples, we calculated TAS/TOS ratio which represents quantitative measure of antioxidative substance and oxidative substance ratio in samples. **Figure 1** shows TAS/TOS ratio for all sample series.

TAS/TOS ratio does not show any significant difference in the same samples before and after TBH addition, but data from the figure obviously show that TAS/TOS ratio is significantly higher ($p<0.05$) in all Med(i)ra samples with and without added TBH, when it is compared with those in serum and Trolox samples with and without TBH.

The only parameter that provides determination of oxidants and antioxidants simultaneously in one single test and shows their balance in sample is PAB. PAB data in all samples are shown on **Figure 2**.

The data from the **Figure 2**. clearly shows that PAB values are significantly lower in all Med(i)ra samples compared to serum or Trolox samples, with or without added TBH ($p<0.05$). Despite that **Figure 2**. shows difference between serum (serum with added TBH) and Trolox (Trolox with added TBH) samples, that difference isn't significant. Also, there is not significant difference in same samples before and after TBH addition.

DISCUSSION

The aim of this study was to estimate antioxidative effects of the supplement based on herbal complex (hydrosol mixture), rosmarinic acid (RA) and honey. The main compounds of this supplement are terpenes and terpenoids, phenolic acid (rosmarinic acid) and other polyphenolic compounds with antioxidative activity.

TAS and SHG showed antioxidative protection capacity, while PAB shows balance between prooxidants and antioxidants in organism. Data collected in this study showed that TAS and SHG levels were significantly higher in all Med(i)ra samples (with and without added TBH), while PAB was significantly lower in these samples compared to serum (with and without added TBH) and Trolox samples (with and without added TBH), despite Trolox antioxidative activity. This suggest that Med(i)ra, despite addition of TBH, has high antioxidative capacity. Preliminary *in vitro* study preformed in our laboratory, which tested antioxidative activity of eight aromatic plants hydrosols (which include rosemary, cade juniper, fennel and lavender hydrosols) with and without added TBH, shows that hydrosols didn't significantly changed TAS values in samples without TBH addition, but TOS was significantly lower in samples with rosemary and cade juniper hydrosols

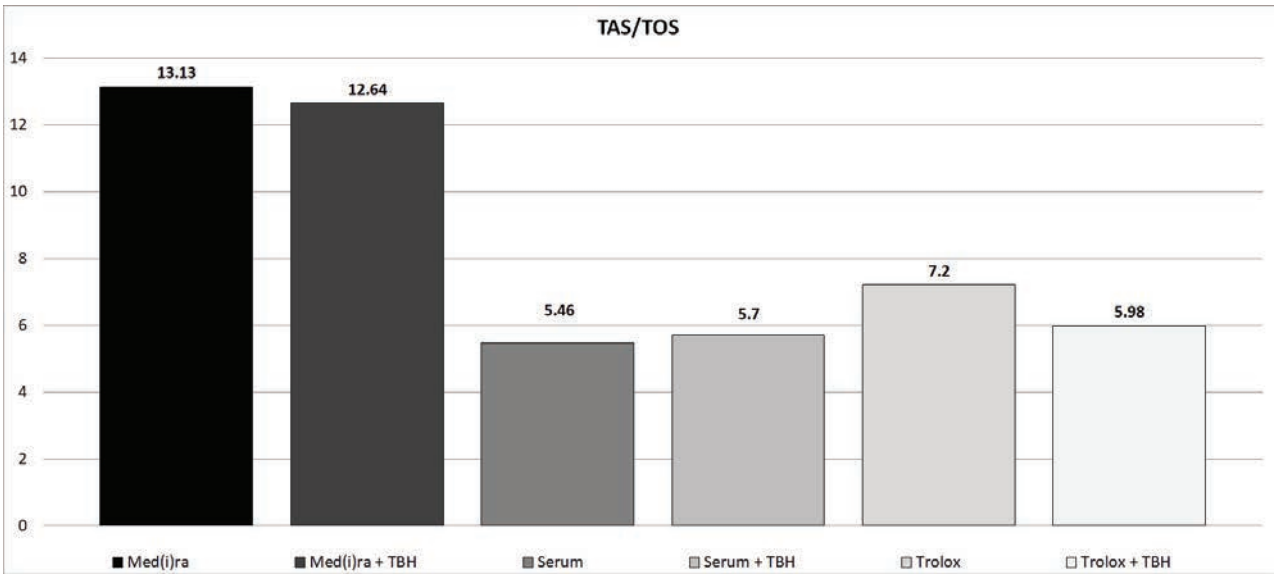

Figure 1. TAS/TOS ratio in all sample series

*p values between same groups with and without added TBH are showed on graph; a – Med(i)ra vs serum; Med(i)ra vs Trolox; b-Med(i)ra with added TBH vs serum with added TBH; Med(i)ra with added TBH vs Trolox with added TBH; a,b - $p<0,05$ (compared using non-parametric Mann-Whitney U test).

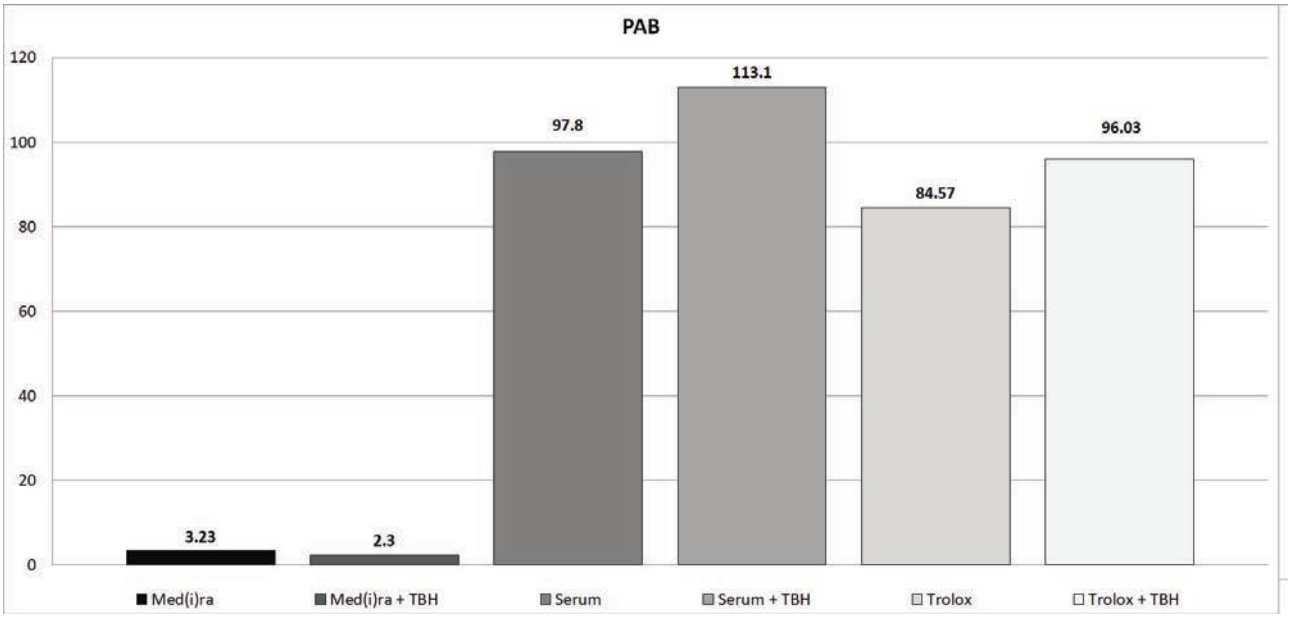

Figure 2. PAB values in all sample series

*p values between same groups with and without added TBH are showed on graph; a - Med(i)ra vs serum; Med(i)ra vs Trolox; b – Med(i)ra with added TBH vs serum with added TBH; Med(i)ra with added TBH vs Trolox with added TBH; a,b – $p<0,05$ (compared using non-parametric Mann-Whitney U test).

(unpublished results). After TBH addition in all hydrosol's samples, TAS value of lavender hydrosol sample was significantly higher compared to fennel and cade juniper hydrosol samples, while TOS was significantly lower compared to serum sample, which lead to higher TAS/TOS ratio values of lavender hydrosol samples. Also, TAS/TOS ratio of lavender hydrosol sample was significantly higher compared to cade juniper hydrosol sample. Rosemary hydrosol significantly decreased TOS and AOPP values compared to their values in serum and showed the best antioxidative capacity compared with other hydrosols used in this study. All data in this study suggests that rosemary, cade juniper, lavender and fennel hydrosols have antioxidative potential [22]. The other study showed that rosemary hydrosol has the ability to act like scavenger of hydroxyl radical, while lavender hydrosol has great potential to act like superoxide anion scavenger [20]. All of these indicate that RA has a great role in Med(i)ra's antioxidative activity. Many studies, including study that investigates antiapoptotic and antioxidative effects of RA on astrocytes [23] and also study that researched protective effect of RA on hydrogen peroxide-induced apoptosis of neural cells [24], showed that RA has a great antioxidative capacity. In study, that compared antioxidative activity of hydrosols to antioxidative activity of hydrosols with added RA, has been shown that TAS/TOS ratio was significantly higher in samples after the RA addition, which suggests that RA significantly improves antioxydative activity of hydrosols [25]. In this case RA probably works as a ROS "catcher" by hydrogen donating and, in that way stabilizes newly formed free radical structure [11].

In addition to significantly higher TAS and SHG levels, and significantly lower PAB in Med(i)ra samples without added TBH, Med(i)ra provides significantly higher PON1 levels and significant decrease of AOPP levles compared to those in serum or Trolox samples. *In vivo* study in estrogen-deficient rats shows that RA has a capability to decrease AOPP values in rat's blood. It is assumed that RA leads to decreasing of AOPP value by inhibition of myeloperoxidase, which catalyzes reaction between chloride ion and hydrogen-peroxide. This reaction leads to hypochlorous acid production, which may induce the AOPP formation [26]. However, after the TBH addition in all samples, AOPP and PON1 values remained lower in Med(i)ra's samples compared to serum and Trolox samples, but that difference wasn't significant (**Table 3**). All of these indicate that Med(i)ra can increase antioxidative activity of PON1 enzyme and decrease the protein oxidation to some extent. In study, which examined the protective effects of RA on the memory impairment in a mouse model induced by amiloid beta protein (Aβ), it has been shown that RA decrease protein damage, induced by protein nitration, by acting like peroxynitrites scavenger [27].

In this study, TAS and SHG levels were significantly higher and PAB was significantly lower in Med(i)ra samples compared to Trolox samples, while TOS levels was significantly lower in Trolox samples (**Table 2**). Other studies also showed that RA provide more efficient antioxidative effect compared to Trolox and other forms

of vitamin E, like γ-tocopherol. However, the most efficient antioxidative effect was gained by synergistic action of RA and vitamin E. RA probably achieves synergistic effect with vitamin E by decreasing vitamin E oxidation, rather than regeneration, like other antioxidants that act synergistically with vitamin E [30, 31]. When TAS, TOS and PAB values and TAS/TOS ratio of Med(i)ra samples were compared before and after TBH addition, no significant difference was observed. That indicates that Med(i)ra can keep its antioxidative potential despite TBH addition. After TBH addition only SHG value was significantly lower, while AOPP value was significantly higher. Beside significant decrease of SHG value in Medi(i)ra samples after TBH addition, that value was significantly higher compared to those values in serum and Trolox samples with added TBH. In addition to studies that showed antioxidative effects of hydrosols and rosmarinic acid [11,12,14,15,20], study performed on endothelial cells showed significant antioxidative protection of honey in presence of various prooxidants. Honey protects GSH as well as regenerates GSH from GSSG, which is, most probably, effect of honeys lipophilic components [30].

CONCLUSION

Results of this study showed that Med(i)ra significantly increased concentrations of antioxidative parameters TAS and SHG, decreased concentration of prooxidative/antioxidative balance parameter (PAB), while significantly increased TAS/TOS ratio, even with added TBH. In this *in vitro* study Med(i)ra also affected PON1 and AOPP parameters, by which it proved itself to be a very efficient antioxidative agent. From all of the above, it is clear that antioxidative activity originates from synergistic effect of this supplements antioxidative components (hydrosols, rosmarinic acid and honey).

Because of the supplement's complex composition, as well as metabolic changes of the supplement's components due to its *in vivo* admission, further *in vivo* studies are necessary in order to test this supplement's antioxidative potency within organism.

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In vitro ispitivanje antioksidativne aktivnosti preparata ruzmarinske kiseline i meda

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Kratak sadržaj

Usled narušavanja ravnoteže između slobodnih radikala i antioksidanasa, dolazi do oksidativnog stresa, patofiziološkog stanja, koje je povezano sa nastankom i razvojem mnogih oboljenja. Kako su se određeni sintetski antioksidansi pokazali kao potencijalno štetni po zdravlje ljudi, sve se više pažnje usmerava ka pronalaženju prirodnih antioksidanasa. Polifenoli, fenolne kiseline, u koje spada i ruzmarinska kiselina, terpeni i terpenoidi su sekundarnii metaboliti biljaka koji poseduju antioksidativne osobine, a mogu se naći u različitim biljnim kompleksima (hidrolatima) i medu. Cilj ovog rada je utvrđivanje in vitro antioksidativne aktivnosti preparata koji predstavlja mešavinu biljnih kompleksa (hidrolata), ruzmarinske kiseline i meda. U „pool" seruma, koji je dobijen mešanjem seruma zdravih pojedinaca, dodavana su serijska razblaženja (100%, 50%, 25%) ispitivanog preparata, kao i serijska razblaženja ispitivanog preparata (100%, 50%, 25%) u prisustvu terc-butil hidroperokisda (TBH), kao prooksidansa. Pored ovih uzoraka, pripremljeni su i uzorci u koje je dodavan troloks (hidrofilni analog vitamina E), kao i troloks u prisustvu TBH. Iz svih uzoraka vršeno je određivanje parametara antioksidativnog stresa: totalnog antioksidativnog statusa (TAS), paraoksonaze-1 (PON1), ukupnog sadržaja sulfhidrilnih grupa (SHG), totalnog oksidativnog statusa (TOS), prooksidativnog-antioksidativnog balansa (PAB) i uznapredovalih produkata oksidacije proteina (AOPP). Takođe, izračunat je i odnos TAS/TOS koji predstavlja kvantitativnu meru odnosa antioksidativnih redukujućih supstanci i oksidujućih supstanci u serumu. Poređenjem vrednosti antioksidativnih parametara TAS, SHG, TAS/TOS odnosa i parametara ravnoteže prooksidanasa i antioksidanasa PAB za uzorke preparata bez i sa dodatkom TBH, nije uočena značajna razlika, što ukazuje da je i pored dodatka TBH ispitivani preparat održavao svoje antioksidativno delovanje. Ispitivani preparat pokazao je značajno antioksidativno delovanje, što se može pripisati sinergističkom delovanju terpena i terpenoida iz hidrolata, ruzmarinske kiseline, polifenola i ostalih antioksidativnih supstanci iz meda.

Ključne reči: *Oksidativni stres; Antioksidans; Hidrolati; Terpeni; Polifenoli; Ruzmarinska kiselina; Med.*

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Stručna izdanja:

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