Current challenges for modern vaccines and perspectives for novel treatment alternatives

Karen Alejandra Garduño-González[1], Samantha Ayde Peña-Benavides[1], Rafael G. Araújo[1*], Carlos Castillo-Zacarías[2], Elda M. Melchor-Martínez[1], Mariel Araceli Oyervides-Muñoz[1], Juan Eduardo Sosa-Hernández[1], Saul Purton[3], Hafiz M.N. Iqbal [1*], Roberto Parra-Saldívar[1*]

[1]Instituto Tecnológico y de Estudios Superiores de Monterrey, Engineering & Science School, Monterrey, Nuevo León, México.

[2]Universidad Autónoma de Nuevo León, Facultad de Ingeniería Civil, Departamento de Ingeniería Ambiental, Ciudad Universitaria S/N, San Nicolás de los Garza, Nuevo León, C.P. 66455, México

[3]Algal Research Group, Institute of Structural and Molecular Biology, University College London, Gower Street, London, WC1E 6BT, UK.

* Correspondence:
rafael.araujo@tec.mx (Rafael G. Araújo); hafiz.iqbal@tec.mx (Hafiz M.N. Iqbal); r.parra@tec.mx (Roberto Parra-Saldívar)

Abstract

With the on-going pandemic, vaccine developing methods have gained attention of the scientific community, specially towards the production, downstream and transport aspects, making it clear that new methods with less complex production and transport are needed, especially for developing countries. In this work we review the current methods used for vaccine production, downstream platforms, and distribution aspects along with the challenges faced by each of the approaches. Some studies have also been carried out   proposing alternatives, the most attractive one being the concept of edible vaccines, which suppose a considerable expenditure cut for the production and distribution of vaccines, in this work we also review some of them, using mainly algae, yeast and bacteria. Algae, yeast and some bacteria have been granted the GRAS (Generally Recognized As Safe) state by the FDA and European food safety authority, making them ideal and safe as vaccine vectors and biofactories at the same time.
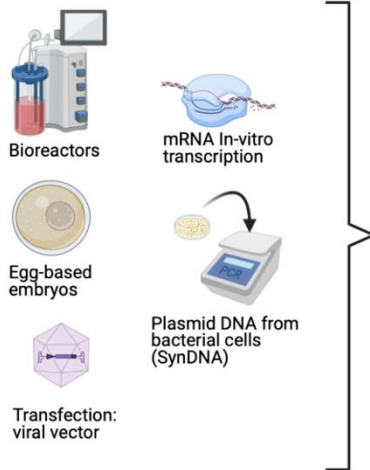
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35 **Graphical Abstract**
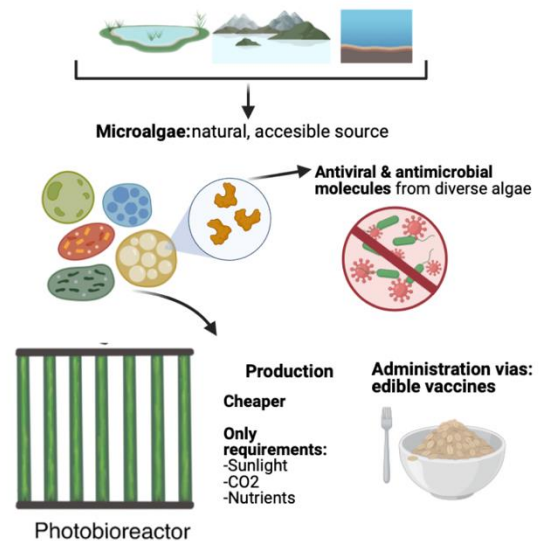
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37 Original image made with the online Biorender program.

Abbreviations list

Abreviation	Meaning
HPV	Human Papillomavirus
HIV	Human immunodeficency virus
SARS	Severe a cute respiratory syndrome
HeV	Hepatitis E virus
MERS	Middle East Respiratory Syndrome
CoV	Coronavirus
LASV	Lassa mammarenavirus
RVFV	Rift Valley fever virus
CCHF	Crimean–Congo hemorrhagic fever
CSFV	Classical swine fever virus
HAdV	Human Adenovirus
HSV	Herpes simplex virus
VZV	Varicella-zoster virus
VACV	Vaccinia virus
SPV	Shope papilloma virus
RV	Rhinovirus
DENV	Dengue virus
JEV	Japanese encephalitis virus
YFV	Yellow fever virus
ZIKV	Zika virus
RuV	Rubella virus
EV-A71	Enterovirus A71
HAV	Hepatitis A virus

CHIKV	Chikungunya virus
RRV	Ross River virus
MeV	Measles virus
PPR	Peste des petits ruminants
NDV	Newcastle disease virus
MuV	mumps virus
RsV	Respiratory Syncytial Virus
VSV	Vesicular stomatitis virus
VHSV	Viral hemorrhagic septicemia virus
EBOV	Ebola virus
HTNV	Hantaan orthohantavirus
HBV	Hepatitis B virus

1. Introduction

The current global pandemic caused by the new coronavirus strain, SARS-CoV-2 which originates the COVID-19 disease, has driven the attention of investigators towards actual vaccine developing methods, available platforms, production and development costs, time and production availability worldwide. The importance of this issue is based on its preoccupant recurrence, as this is the third documented human-animal virus outbreak in a fairly short time span of 20 years [1]. Immunization is one of the best if not the most effective strategy for preventing an infectious disease and keeping control of many important viral pathogens. Viral vaccines induce immunity before a viral infection takes place, their mechanism relies basically on adaptive immune responses for protection, which are triggered once the immune system detects and reacts against the viral particles introduced by the vaccine. The success of immunization depends on the efficiency of antigen recognition, expansion, memory, trafficking and the numerous functions of lymphocytes [2,3].

Historically successfully vaccine-induced immunity has determined the spread and maintenance of a viral pathogen within certain population, for example the world-wide eradication of smallpox allowed society to develop to the point we stand now, it had been a serious health problem for approximately 3000 years and killed over 300 million people in the 20th century [2,4].

Despite the great immunology advances, infectious diseases are still one of the most important threats to public health, in the last decades there has been an important increment of new human pathogens, most of them, at least 70% being from zoonotic precedence. Some examples of this are HIV, avian influenza, HeV and Nipha; most recently we have seen the rise of several new zoonoses such as SARS, MERS-CoV, Ebola, Marburg, LASV, RVFV and CCHF and of course novel SARS-CoV2, which have

61   represented quite a challenge for immunologists as the viruses spread rapidly in our global society due

62   to the increased urbanization, international travel, commerce and climate change increase the

63   probability of emerging pathogens, as far as we know zoonosis will continue and even worsen in the

64   future [2–7].

65   Zoonotic viruses take advantage of new human hosts due to their scarce or no pre-existing immunity,

66   giving   the virus an open pat   hway to enter   and replicate inside receptive cells, evading clearance

67   by the host immune system for long enough to be transmitted to another susceptible host. The lack of

68   herd immunity results in a quite quick viral dissemination [2,7].

69   This situation highlighted several current challenges to overcome, such as the lack of information about

70   correlates of protection, antigenic variability or immunodominance; one prominent study that needs to

71   be carried out is the development of an appropriate animal model of the disease   it is also important

72   to consider that time is key in order to stop the spread, the quicker the vaccine can be developed, the

73   less infected hosts [2,8].

74   Along with the great advances that vaccines represent for humankind, economically, they are one of

75   the best investments available, giving a return at least 16 times greater than the inversion needed and

76   at best yields an average of $44 (US dollars) in economic returns, these results are taken from a societal

77   perspective using the cost-of-illness approach and considering immunized children grow up healthy

78   and can achieve their full potential. Still, this ROI (return of investment) depends upon investing the

79   necessary amount for national immunization programs [4,9]. Global immunization programs are vital

80   for our survival, so goes hand in hand the insurance of vaccine's stability [10].

81   Generally, a vaccine is a particle that can generate an immunological response that eventually derives

82   in long-term protection against the pathogen from which the molecule proceeds or resembles, this

83   immunization process is depicted by Figure 1. Also pictured on Figure 1 are the 7 most relevant vaccine

84   types, and the current production platforms which will be discussed in the present work along with

85   some edible vaccine's alternatives.

86   The main focus of this revision is to elucidate the current status of the vaccine industry, the current

87   production methods and downstream processes along with their principal characteristics, challenges,

88   most recent technology and future perspectives and alternatives based on unicellular microorganisms.

89

90   **2. Vaccine Types**

91

2.1 Live attenuated vaccines

Live attenuated vaccines are derived from wild viruses or bacteria, attenuated or weakened usually by repeated culturing. Live attenuated vaccines in liquid formulations are highly unstable due to the need to maintain viability of the pathogen. These vaccines, as the protein based, are susceptible to chemical and physical destabilizing processes, as pathogens' structural proteins and glycoproteins can be compromised, affecting viability, infectivity, immunological response and vaccine effectivity. Depending on the stability of each pathogen facing different environments the final presentation is chosen, i.e. enveloped viruses are more labile than nonenveloped due to the bilayer of their envelope, making them susceptible to damage in aqueous solution, thus most live vaccines are freeze-dried [11].

The potency of live-attenuated vaccines (measles & yellow fever) can drop really quick, once reconstituted [12], thus the WHO recommends for these to be kept cold and discarded after 6h. This policy is based on the instability and the minimization of the chance of bacterial contamination as live vaccines do not contain preservatives [13].

Nevertheless, there are several vaccines whose formula has already been enhanced to cope with greater temperatures, commonly found at room temperature, such is the case of heat stable CSFV, this new formulation, ST16 containing excipient combinations of trehalose, glycine, thiourea and phosphate buffer, proved to be safe and effective when immunized to piglets *in vivo*. This new formulation proved to have a better performance under high temperature conditions (37-45 ℃) [14].

Another example is the rotavirus vaccine is another example, the disease is mainly reported in developing low-income countries and has priority in their national immunization programs, since these countries have several limitations for effective storage, to help mitigate the need of cold chain related issues, SIIPL developed a thermostable rotavirus vaccine, ROTASILL® which amplifies the common storage temperature below 25 ℃ for 36 months and tolerates temperatures of 37-40 ℃ for 18 months and short-term exposure up to 55 ℃, it also survives a temperature shock of being thawed from -20 to 42 ℃ [15].

2.2 Inactivated vaccines

All inactivated viral vaccines start by pathogen cultivation on a substrate to produce large quantities of antigen. Eggs, cells, tissues and even whole living systems have been used as substrates for this purpose, recently there has been a shift to growth on continuous cell lines. After propagation the virus is harvested, purified and concentrated, followed by chemical or physical inactivation. Some examples

123   for inactivation are formaldehyde and ß-propiolactone (BPL) which have been widely used for licensed

124   human viral vaccines [16]. This kind of vaccine has   been developed for influenza, hepatitis A,

125   rabies, polio and encephalitis, they are injectable, administered intramuscularly.
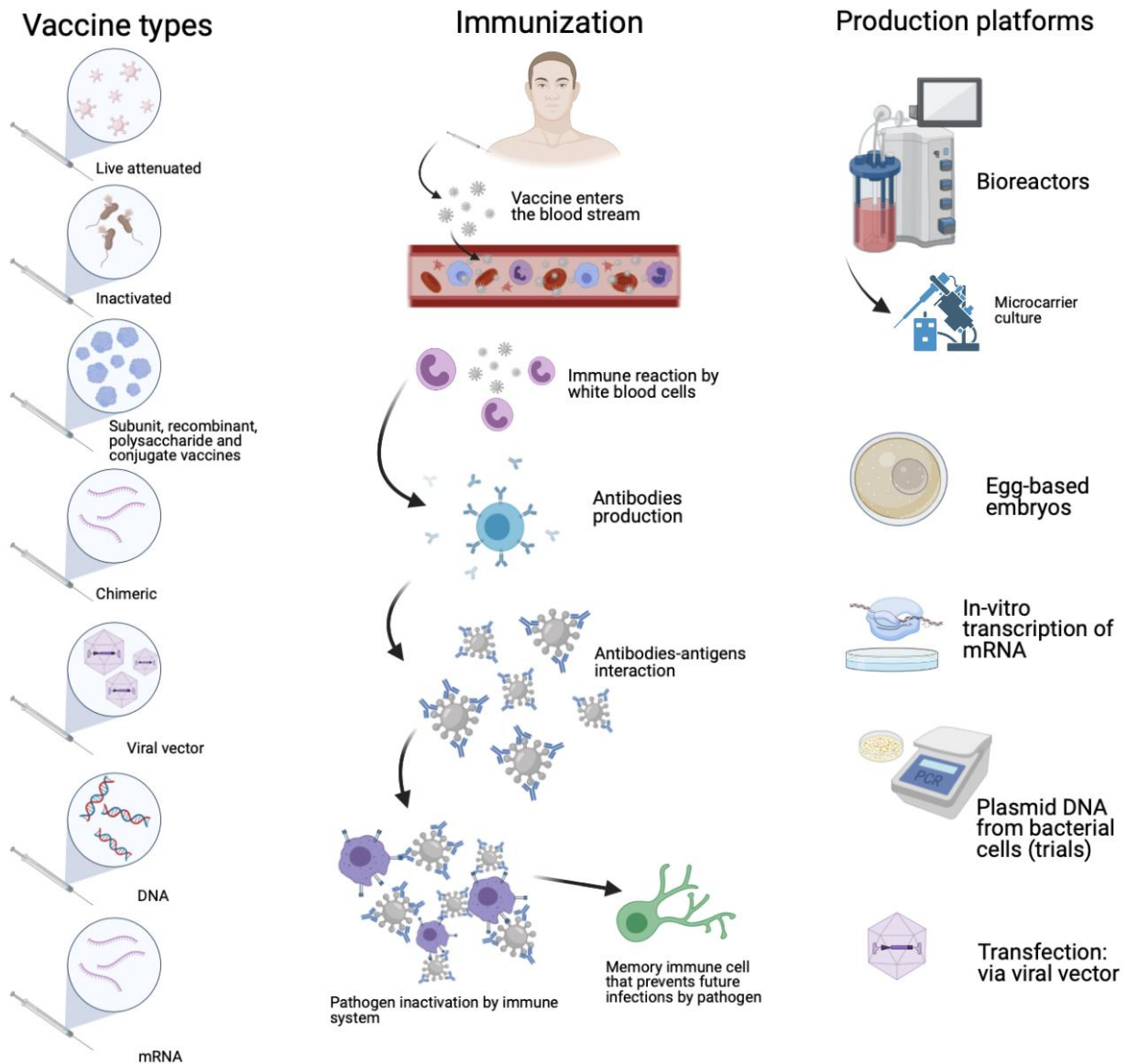
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127   About stability, they are more stable during long-term storage and are developed as liquid formulations

128   stored in glass vials or prefilled syringes, still they are sensitive to freezing and susceptible to potency

129   loss during storage and distribution [17].

130

131 **Figure 1.** Vaccine types, immunization process and current platforms. Original image made with the
132 online Biorender program.

133

134

135

136 **2.3 Virus-like particles**

137 **2.3.1   Subunit vaccines**

138 Subunit vaccines are made by extracting and purifying some components of the bacteria that can trigger
139 an immune reaction [18]. They are safer than attenuated vaccines because there is no risk of subsequent
140 reversion and are less thermally sensitive as there is no need of keeping an organism alive [18,19].
141 In the downside, these vaccines can fail to initiate an efficient immunologic process, so it has to be
142 administered to patients in several doses at a specific timing to ensure their long-term efficiency [20].
143 Then again, addition of adjuvants is key to get the desired immune response. Here is where most of
144 stability issues begin, formation of aggregates due to aluminum addition [21].

145

146 **2.3.2   Recombinant vaccines**

147 Vaccines derived from DNA recombination technology   are obtained by inserting DNA fragments
148 to encode the desired antigens into bacterial, yeast, or mammalian cells and then antigens are expressed
149 in cells, extracted and purified to be administered to patients. This vaccine type can reduce the risk of
150 virus and toxoids reversion and can produce greater amounts of antigens. These vaccines must be
151 highly specific to get an adequate immune response, moreover different kinds of adjuvants are needed
152 to ensure their stability. There are several strategies for this technique, like recombination of proteins,
153 live vectors and injecting foreign naked DNA into an organism to produce an immune response [22].
154 Depending on the approach of the vaccine, different stability issues arise. The most common issue
155 between them is the adjuvants needs and their role in the stability. Vaccines contain aluminum salts as
156 adjuvants which affect stability during cold chain. Although heat is not the problem, as it can be
157 exposed to 45 ºC for a week or 37 ºC for a month and still hold immunogenicity, when shifting to
158 lower, freezing temperatures the sensitivity of the vaccine is relevant as aluminum salts may aggregate
159 during the thawing process, which may cause irritation at the injection site and reduce the potency. To
160 improve this situation, phosphate buffer has been added as the surface of the aluminum adjuvants
161 changes and prevents agglomeration, as well as the addition of polyethylene glycol that contributes to
162 the depression of the freezing point. [23–25]. PS80 (polysorbate 80) has also been used as a surfactant
163 along with electrolytes to protect the surface of the virus like particles while electrolytes provide
164 sufficient ionic strength [17].

165

2.3.3   Conjugated polysaccharide vaccines

Polysaccharide capsular antigens from bacteria like *Haemophilus influenzae* type b, produce an effective serum antibody response when used as a vaccine. A polysaccharide hapten covalently conjugates with a protein carrier, it triggers a humoral immune response. In these polysaccharides' DNA bases are linked to a deoxyribose backbone or carbohydrate monomers by glycosidic bonds, so opposite charges between antigen-adjuvant are necessary for a useful formulation [17].

Ionic strength has a significant effect on   stability. Lower ionic strength (below 0.15) with higher salt concentrations leads to lower biomolecular solubility. Also, anions and cations show tragic effects, inflating macromolecular solubility, affecting intra and inter molecular stability [17].

2.4 Viral vector vaccines

This technology relies on the use of a modified virus, different from the pathogen of interest, for the delivery of one or more antigens encoded in the unrelated virus which can be alive and attenuated or non-replicating vectors. This is a quite versatile platform, there are several engineered viruses as vectors to encode for heterologous antigens that are shuttled into the host cells, once they get delivered and expressed, the host detects them, and the immunological cascade starts [8,26].

These vectors need to be viable when administered to carry out their labo   r, which makes them quite sensitive to any kind of stress, the formulations require some adjuvants to maintain effectiveness, for long-term storage liquid and lyophilized powder must be stored at -70 ℃ and could also be stored for up to 1 month at 2-8 ℃. Again, the excursions outside the cold chain could lead to important potency losses. This makes a bit difficult the distribution and maintenance of the vaccines in developing countries, especially those with hot weather [27].

2.5 DNA vaccines

DNA vaccines, consisting of a vector with a eukaryotic cell gene and promoter, encoding an immunogenic protein, have been shown to cause a robust cytotoxic T cell response compared to subunit vaccines. They have the ability of inducing both cellular and humoral immune responses, but they also have a low transfection efficiency and perform poorly in clinical trials, requiring booster doses to achieve desired immune response, on the other hand they also can be administered to immunocompromised patients, which gives them a great advantage [28].

Compared to protein vaccines, DNA vaccines production platforms have lower cost and enhanced stability for transportation, storage and distribution as they do not require such rigorous temperature

198 control, still the stability of nucleic acid-based vaccines under dry conditions or aqueous conditions is

199 highly dependent on stabilization techniques.

200 It is important to note that this technology still has some important delivery-related issues to address,

201 related to the safety of the hosts genetic material, it is possible that some of the vaccine's DNA gets

202 mixed into the host's, along with its low tolerance to temperature changes,   electroporation has been

203 considered in order to improve delivery efficiency [29–31].

204

205 **2.6 mRNA vaccines**

206 mRNA vaccines have a similar   mechanism of action   like DNA vaccines, mRNA capable of

207 encoding for an antigen is delivered to the host, the antigen is produced by host's cellular machinery

208 and then degraded by enzymes. This molecule is non-infectious and non-integrating, ensuring safety

209 for the host against infection and mutagenesis [32].

210 This approach has some important advantages in comparison to other techniques. Regarding

211 oligonucleotides and small molecule drug targets, mRNA can influence the stimulatory and inhibitory

212 mechanism of action; compared to DNA vaccines that need to enter the cell nucleus risking genetic

213 integrity, mRNA only needs to access ribosomal translation machinery; finally in contrast to proteins

214 and viral systems, mRNA manufacturing is way easier and faster as it does not involve   cells, also

215 the proteic product has native glycosylation and conformational properties [33,34].

216 Stability is a major concern regarding the storage temperatures, as they   need a typical storage

217 temperature range of 2-8 ℃ and -70 ℃ for the long term. The design of optimally stabilized mRNA

218 vaccine formulations during storage, transport, and administration at refrigerated or   room

219 temperatures should be addressed first to obtain suitable vaccines for all countries [27].

220

221 **3. Transportation, storage, and temperature related damage to vaccines**

222 Most vaccines are made of proteins, therefore, instability of proteins affects protein vaccines potency

223 directly. Protein's instability in solution can be caused by different chemical and physical processes,

224 most of protein loss is due to the protein unfolding that leads to the alteration of quaternary and tertiary

225 structures with subsequent aggregation of denatured proteins to minimize unfavorable thermodynamic

226 interactions. These events lead to the loss of specific characteristics that made the protein biologically

227 relevant to generate an immune response [10].

228 Chemical instability is the one caused by unwanted reactions such as hydrolysis, loss of functional
229 groups, formation or breakage of disulfide bonds, oxidation and other alterations that modify protein's
230 proper functions. These processes are triggered or influenced by pH, buffer, salts, ionic strength or
231 adjuvants and can be accelerated by temperature   changes [10].

232 Other kind of interference   may also affect stability, for example, agitation of the Hepatitis B vaccine,
233 as would be expected during transportation, causes some vaccines to freeze completely within 3-6 h,
234 which could be a risk factor for adjuvants stability [35].

235 Most vaccines currently available globally are stored and transported under a cold chain system at 2-8
236 ℃ or below 20 ℃ [15].

237 Heat is a key factor to vaccine damage and the most usual. The damage could be the direct result of
238 inadvertent exposure to elevated temperatures, in the case of lyophilized vaccines, heat shock when
239 diluent is too warm when added. The response of each vaccine to heat exposure varies widely. High
240 stability: HPV, diphtheria, tetanus toxoid, and Hepatitis B; moderate stability: freeze-dried measles,
241 yellow fever and BCG; low stability: oral poliomyelitis [12]. Exposure of vaccines to sub-zero
242 temperatures does not necessarily means that there will be considerable damage to the potency, but it
243 is still a high risk for unwanted interactions.

244 As stated by Chen & Kristensen (2009), all vaccines lose potency over time and this loss is mostly
245 temperature dependent. Most used vaccines have a shelf life of 2 years or longer if kept under
246 refrigeration (2-8 ℃). Still, the problem we are facing relies more on the distribution side, the
247 sensitivity of vaccines to temporary temperature shifts outside their validated range varies
248 considerably. Excessive heat should not be considered as the main risk, inadvertent freezing is also an
249 important problem regarding vaccine integrity [36].

250 Modern vaccines are highly dependent on the cold chain to maintain vaccines viable, thus, predicting
251 vaccine stability is also highly important, to maximize vaccine's lifespan under real storage conditions.
252 As an example, we can take the challenges faced for COVID-19 vaccine regarding the cold chain, the
253 main problem being mRNA's sensibility to temperature shifts, thus the lack of proper storage systems
254 across countries and the difficulties for monitoring the vaccine's temperature along transportation were
255 critical [37].

256 Liquid vaccine presentations and formulations have   and continue to be the most straight-forward
257 approach, as injection is the most common administration via and their manufacture and package are
258 relatively easy, most of the developed platforms are designed to deliver liquid final products. Freeze-
259 dried vaccines have only been produced, if necessary, to achieve stability.

260 Meanwhile, the stabilization of proteins in aqueous solution is based on mitigation of the detrimental
261 effect of the constant proton exchange between proteins and the environment, every exchange at the
262 protein's surface leads to a temporary charge change that over time prevails over the first status with
263 functional charge, the new charge status of the protein can lead to aggregation and denaturation. These
264 exchanges can be controlled by specific buffering systems, leading to enhanced stability of the proteins,
265 this has been successfully carried out with Hepatitis B vaccine [38,39].

266

267 **4. Predictive methods to improve vaccines' stability**

268 Formulations resistant to heat damage have major benefits, such as reducing vaccine wastage, ensuring
269 the effectiveness and of course being less dependent on cold-chain supplies makes them easier and
270 cheaper to transport, in addition to making them available for developing countries or in emergency
271 situations when the cold chain might break down [40].

272 Clénet D. (2018) applied a combination of advanced kinetics and statistical analyses on vaccine forced
273 degradation data to accurately describe the loss of antigenicity for a multivalent freeze-dried
274 inactivated virus vaccine containing three variants. The screening of large amounts of kinetic models
275 combined with a statistical model selection approach resulted in the identification of two-step kinetic
276 models. Predictions based on kinetic analysis and experimental stability data agreed, showing that
277 model   ing a few months of forced degradation can be used to predict various time and temperature
278 profiles endured by vaccines such as long-term stability, short time excursions outside the label   ed
279 storage conditions or shipments at room temperature, with high accuracy [41].

280 While prediction kinetics and adjuvants are key to prevent wastage and enhancing vaccines' shelf life
281 and even potency, there are still several points that could be improved and would make production
282 easier, as reviewed before, most of the stability issues are due to the addition of adjuvants which are
283 needed to keep vaccines' stability in liquid and powder mediums, where the molecules of interest have
284 already been purified. In this context, the development of new vaccines technology such as DNA and
285 mRNA vaccines that do not require such strict conditions would represent a simplification in the entire
286 process impacting directly in production costs and even improving efficiency.

287 **5. Current vaccine production platforms**

288 Based on the former stability considerations, there are several factors that researchers have to consider
289 before scaling the production process. Production platforms are designed regarding the nature of the
290 vaccine, its compounds' stabilities, and complexity.

291 For modern vaccines, the most common platforms are mentioned in Table 1. As we can see, most of

292 the existing platforms require considerably specified, expensive installations, while most of them are

293 already being used to manufacture commercial vaccines, some others like the DNA and mRNA

294 technologies are still being developed.

295 Most of vaccines' production cost relies on the platforms being used, the supplies needed, maintenance,

296 high-purity reagents, filters, cold-chain transportation, storage and adequate packaging and of course

297 the dosage needed, for example, annual operating costs required to meet the global demand would cost

298 ≈17 billion USD/year in case of vaccines with 100 μg of mRNA [42].

299 Now, considering the actual global situation regarding COVID vaccines' development and distribution,

300 and taking into account all the implications for vaccine development and further scalability, for

301 conventional vaccine technologies mentioned in Table 1, the most approachable are the ones that have

302 been successfully taken from investigation to commercial production, as most of them have already

303 overcome the most critical issues, such as costs, platform design and product stability. Nonetheless,

304 these technologies do not consider the emergency state now and certainly are not fitted for a situation

305 where low-income and developing countries that may not have the necessary infrastructure to produce

306 new technology vaccines and furthermore would not be able to satisfy their   expensive demand, the

307 need to produce their own vaccines and get them to every   inaccessible places while keeping their

308 viability. The latter would be quite a task as most of the vaccines require strict storage conditions

309 regarding temperature   .

310

311 **Table 1.** Current production platforms for vaccines

Production Method	Virus	Vaccine type	Approach	Commercial vaccines examples	References
Suspension-Vero cell cultures [43] Vero cell expansion on micro-carriers	HAdV, HSV, VZV, VACV, SPV, RV, MERS-CoV, SARS-CoV & -CoV2, DENV, JEV, YFV, WNV, ZIKV, RuV, EV-A71, HAV, CHIKV, RRV, HTNV, Influenza A & B, MeV, PPR, NDV, MuV, RSV, VSV, Reovirus, Rabies virus	Live attenuated, Inactivated, Chimeric	Virus production by attenuation or inactivation and subsequential induction of immune response	Ervebo® Vepacel® Preflucel® IMOJEV® Ixiaro® IMOVAX Polio® OPV® VERORAB® RotaRIX® RotaTeq® ACAM2000®	[43]
Pathogen-free embryonated chicken eggs	Measles	Live attenuated		M-M-R® II	[43,44]
Virus production using a microcarrier	Retrovirus	Inactivated		Advanced investigation stages	[43,45]

				for human T-cell leukemia virus type 1.	
in bioreactor					
Egg-based: 11-day-old embryos are used as substrate	Influenza	Inactivated		450/477 Fluzone ® Quadrivalent	[43,46]
Suspension-cell, Mammalian cell	Hepatitis C	Virus like particles	VLPs isolated from the cell lysates to generate immune response	Investigation stage [47]	[43,47,48]
Baculovirus expression system (BVES)	Papillomavirus, Hepatitis E, Poliovirus, Bluetongue virus, Newcastle disease, SARS coronavirus, Hantaan, influenza type A and infectious bursal disease			Cervarix®	[43,49,50]
Stirred bioreactor: *Haemophilus influenzae* Carrier proteins: *Clostridium tetani, Corynebacterium diphtheriae, Neisseria meningitidis*	*Haemophilus influenzae* (Hib) Type B	Conjugate	Separate production of capsular polysaccharide from Hib, and a carrier protein	PedvaxHIB® ActHIB® Hiberix® Pentacel®	[43,51–54]
Marine organisms culture, microalgae	SARS-CoV2, HIV, DEN, HSV, influenza A & B	Polysaccharide	Viral inactivation: direct interaction, adsorption inhibition, transcription & replication inhibition, activation of host antiviral immunomodulatory system	Currently under investigation and clinical studies	[38,55]
In vitro transcription of target mRNA	SARS-CoV2, anti-tumour (tumor suppression), pseudorabies	mRNA	Translation of mRNA into a viral protein that promotes immunological response, self-assembly approach	Currently under investigation and clinical studies	[56,57]
Plasmid DNA derived from bacterial cells using a synthetic DNA platform and electroporation delivery	SGIV, VHSV, ZIKV, EBOV, MERS-CoV	DNA	Transcription and translation of DNA into a viral protein that promotes immunological response	Currently under investigation and clinical studies	[31,58]
Stirred bioreactor: Recombinant cell culture (mammalian,	Rotavirus, hMPV, HIV-1, HBV	Recombinant protein	Recombinant production of a pathogen's protein, which once	RECOMBIVAX HB®	[38,59]

bacteria, yeast and insects)			introduced into the host induces immunological response		
Transfection via viral vector: delivering a transgene antigen	Hepatitis C, RsV, Borna disease virus	Viral vector	Transcription and translation of DNA into a viral protein that promotes immunological response	Currently under investigation and clinical studies	[38]

312

6. Novel nanotechnology delivery methods

313

Nanomaterials in vaccines' formulations have been studied as enhancers of their efficacy, mainly to address challenges that conventional adjuvants cannot solve. One of them is the generation of protective immunity by antigens, which is quite difficult to obtain when treating immune diseases such as HIV, malaria and tuberculosis. The synthetic nature of nanomaterials confides them malleable structures, clear engineering design rules and the implementation of a complex immunization strategy due to the specific combination of humoral and cellular immune responses these materials can trigger [60].

Engineering of nanomaterials has been focused to the delivery of antibodies into specific key cells and tissues. An immunogenic nanomaterial needs to interact with different types of cells, including antibody presenting cells (APCs), B cells, T cells, neutrophils and macrophages after the vaccine's antigens have been released and processed, thus making it a challenge to accurately engineer the trafficking of a nanomaterial vaccine. In this endeavor is important to control the size and shape of the nanomaterial, its lifespan inside the host, quantity of antigen copies on or inside the nanomaterial, co-delivery adjuvants, physical orientation of antigens or complementary activation [61].

An important strategy to enhance immunogenicity of a vaccine is to increase the persistence of antigens at the injection site, in the circulatory system, within the APCs and in lymphoid tissues. This can be achieves by encapsulation or conjugation of antigens with nanomaterials; this was also the effect of prolonged presence of antigens due to the continuous release using poly(lactic-co-glycolic acid) (PGLA), also the degradation rates of this nanomaterial could be tailored to extend or shorten antigens' release, this allows PGLAs to become a durable source of antigen for APCs to get and present to helper T cells [62].

Nanomaterials can also be designed to be sensitive to the environment of some metabolic pathways, mimicking some viral infections and enabling better responses from specialized T cells compared to the free antigen delivery approach. This also contemplates the strategy of controlled and predictable delivery of the nanoparticles to B, T, follicular dendritic and macrophage cells inside the lymph nodes (LN). The LNs are the ones in charge of the long-lived humoral immunity so it is important to mention that the larger the molecules, the better they are retained in the LN and different nanomaterial platforms produce different particles with slight variations of optimal size for lymphatic drainage [60].

Viruses and bacteria present curiously spatially repetitive structures, and the immune system has developed the ability to recognize and act on them with special precision. Regarding the display of proteins on nanomaterials, the possibility of fine manipulation of the nanomaterial gives the chance of reproducing these repetitive structures to get an immune response without any real threat nor supplemental adjuvants [60,63].

The mucosal immune response is relevant in respiratory, sexually, and orally transmitted diseases, thus mucosal delivery of nanomaterials has been the main approach [60].

The latest nanoparticles studies have been focused on the efficient delivery of mRNA and molecules, due to the present pandemic. In this context, most of these have been applied to the mRNA vaccines as those are the ones showing the most promising results for immunization [33].

Lipid nanoparticles (LNP) function as adjuvants for mRNA delivery. mRNA vaccines combined with a lipid nanoparticle delivery system present similar nano-structural properties to viral systems in terms of delivery. Endogenous anionic lipids combine with cationic ones to produce non-bilayer structures resulting in disruption of the endosomal membrane and release of the genetic material into the cell cytoplasm, these lipids resemble to the normal circulating endogenous lipid-containing chylomicrons in terms of size and uptake [34,64].

Cationic polymers as DEAE-dextran, polyethyleneimine, chitosan and Poly(ß-amino esters) have been used to form cationic polyplexes electrostatically bound to nucleic acids, these cationic polymers are made for mRNA and nucleic acids delivery, nonetheless this technology is not as advanced as the LNP mentioned before [33].

7. Novel antiviral treatment prospectives for future development targeting low-income countries

365 Injectable formulations, reviewed above, are biologically safe and sterile, but their need of a cold chain
366 system for their transportation and storage along with the elevated manufacturing costs opens a new
367 road for a generation of vaccines with the ability to withstand room temperature, are cost-efficient,
368 easier to transport and to administer while producing and conserving efficiently the needed amount of
369 antigens to confer immunity to the targeted organism. In the case of edible/oral vaccines, they must
370 also survive the rough environment of the gastrointestinal trac   t.[65]

371 Under this light, edible vaccines are considered one of the main new approaches for administering
372 novel vaccines, due to their convenience as there is no need of trained personal for administration (self-
373 administrable) and manufacturing costs are quite low compared with traditional vaccines, as there is
374 no need for purification or stabilization, they are stable once freeze-dried and maintain the required
375 bioavailability to generate a useful immune response. [65]

376 Oral vaccines elicit immunity via the gut-associated lymphoid tissue, offering   mucosal protection,
377 local and systemic immune responses, which leads to the effective eradication of pathogens. The
378 intestine is one of the immunological organs of the human body, it acts as the first line of protection,
379 thus most produced antibodies are secreted into the gastrointestinal tract.

380 Regarding their mechanism of action, according to Rosales-Mendoza (2016) , the administered
381 vaccines are transported into the Peyer's parches by the M cells and presented to T-cells by the antigen
382 presenting cells. This leads to the growth and proliferation of B lymphocytes, which later differentiate
383 to plasma cells. It is important to note that the achievement of a balance between immunogenicity and
384 mucosal tolerance prevents unnecessary immune responses in the gut mucosa and therefore is
385 necessary to know the tolerated dosage before administration. [66,67]

386 Considering this mechanism of action, food-grade organisms: plants, insects, bacteria and algae have
387 gained more interest in the scientific community for the development of oral/edible vaccines. This
388 approach is focused on offering better efficacy, lower production and administration costs as well as
389 the simplification of the whole vaccination process.

390 To date, most of the edible vaccines have been developed as attenuated vaccines, thus the risk of
391 reversal to pathogenic form is still high. Other edible vaccines that are close to commercialization have
392 been based on plants and are not appropriate for edible delivery, hence the purification of the proteins
393 of interest is necessary for the final formulation. In the case of algae-based vaccines, the yield of
394 production of the desired protein, usually a recombinant epitope, needs to be improved to get an
395 effective dosage-biomass ratio against diseases.

7.1 Microalgae-based vaccines as new production platforms and carriers

Microalgae have been considered as possible vehicles for edible vaccines, as they are considered a food ingredient by the EFSA (European food safety authority), also some of them have the GRAS status (Generally Recognized As Safe) by the FDA: *Arthrospira platensis, Chlamydomonas reinhardtii, Auxenochlorella protothecoides, Chlorella vulgaris, Dunaliella bardawil, and Euglena gracilis* [68]. This status assures that these organisms are safe for human consumption and therefore do not contain any endogenous toxins. Edible vaccines are characterized by being immunologically active,   inducing an immune response in the host and increasing its resistance to a targeted pathogen [69].

7.1.1 Non-transgenic, microalgae-based edible polymers as adjuvants and potential vaccines

Microalgae and cyanobacteria are part of a group of unicellular microorganisms found in aquatic and terrestrial environments, including fresh and sea   water, they have the ability of growing photoautotrophically or heterotrophically, also have unique metabolic pathways for carotenoids, polyunsaturated fatty acids, proteins and polysaccharides production. Some of these compounds present antiviral activity that is further compared and detailed in Table 2 [70,71]. Algae are great hosts for the production of bioactive compounds, some of the most studied include: lectins, fucoidans, polysaccharides and proteins. [72]

Algal polysaccharides are natural polymers,   nontoxic, cheap, biodegradable, and biocompatible. They have been tested for their antiviral efficacy against many viruses including human immunodeficiency virus (HIV) and dengue virus (DENV)   . Thus, they have acquired importance in biomedical and pharmaceutical industries that can be further explored to develop drug molecules targeting SARS-CoV-2   [73].

The latter is one of two approaches for edible vaccines, taking the microalgae as a bio factory and using the molecules they naturally synthesize as antiviral molecules, some to enhance the immunogenic response and others as epitopes.

Regarding the general aspects of developing vaccines using algae, the major advantages are their fast growth rate and therefore biomass production, high post-translational modification capacity, great performance as adjuvant producers and the vast industrial production experience with them. On the

427 other hand, there are not much genetic and glycoengineering tools to manipulate them, which affects

428 the production yields of the proteins of interest when it comes to modified strains.[66]

429

430 **Table 2.** Microalgae polysaccharides, mechanisms of action and current tests as adjuvants and active
431 ingredients for vaccines

Compound	Type	Microalgae	Effects/mechanism of action	Tests	References
Carrageenan	Sulphated polymer	Red: *Chondrus, Gigartina, Hypnea & Euchema*	Blocks the entry of viruses by inhibiting their binding & incorporation to the cell	HPV prevention: HeLa cells & mouse models	[73,74]
Alginates	Polymer containing linear co-polymers of beta-(1,4) linked D-mannuronic acid & beta-(1,4) linked L-guluronic acid	Brown: *Laminaria, Ascophyllum, Macrocystis*	Inhibits viral replication (HIV-1) by decreasing the reverse transcriptase activity, interrupting virus internalization and improving defense mechanisms of the host cell, robust attachment of virus gp120 protein with CD4 molecules on the surface of T cells.	Preclinical: As adjuvant. New drug 911 derived from alginate polysaccharide against HIV-1 at both chronic infection of H9 cells and acute infection of MT4 cells *in vitro* and *in vivo*.	[73,75]
Galactans	Polysaccharides with linear chains of galactoses	Red: *Agardhiella*	Inhibits viral replication (HIV, DEN, HSV) reducing the reverse transcriptase activity and the syncytium formation between infected and uninfected cells	*In vitro*: against dengue virus using Vero cells	[73,76]
Fucans (sulphated)	Polysaccharides, strongly anionic, HMW	Brown: *Dictyota, Lobophora, Fucus, Spatoglossum*	Blocks reverse transcriptase activity	*In vitro*: evaluated using activated DNA, against HIV using poly(rA)-oligo(dT) as template	[73,77]
Nostoflan	Acidic Polysaccharide	Blue-green: *Nostoc*	Inhibits initial stage of virus infection, including virus binding & internalization processes.	*In vivo* using Vero cells, against HSV-1, HSV-2, human cytomegalovirus, and influenza A virus (IAV)	[73,78,79]
Calcium spirulan	Spirulina: sulfated polysaccharide, termed calcium spirulan (Ca-SP)	*Spirulina*	Inhibits virus entry into host cell & syncytium formation (even with low concentrations).	*In vivo*, inhibitor of different viruses, including HSV-1 (in HeLa cells), HCMV (in HEL cells), influenza A (in MDCK cells), Coxsackie virus (in Vero cells), measles (in Vero cells), HIV-1 (in MT-4 cells), polio (in Vero	[75,80]

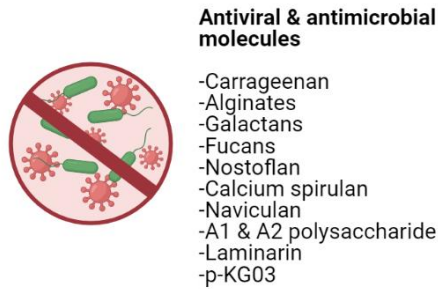|			cells), and mumps (in Vero cells)		
Naviculan	Sulphated polysaccharide (galactose, xylose, rhamnose, fucose, mannose & sulphate)	*Navicula directa*	Inhibits the fusion of cells that express CD4 receptor and HIV, inhibits the initial stages of viral replication, possibly by blocking viral internalization into host cells.	*In vivo* using HeLa cell line, against HSV-1 and HSV-2 and influenza virus.	[73,75]
A1 & A2 polysaccharide	Sulphated polysaccharide (extracellular)	*Cochlodinium polykrikoides*	Not yet elucidated	*In vivo* using Hep-2 cells, against cytopathogenic effects of HIV- 1 in MT-4 cells, influenza virus types A and B in MDCK cells, and respiratory syncytial virus types A and B	[73,75,81]
Laminarin	1) Glucose residues 2) Terminated by D-mannitol	Brown sea weeds: *Laminaria japonica, Eclkonia*	Inhibits reverse transcriptase expression, prevents HIV activity by inhibiting the HIV entry on human-derived lymphocytes and the ability of HIV reverse transcriptase activity, which plays an important role for the virus proliferation	*In vivo* using human lymphocytes, against HIV.	[73,82]
p-KG03	Sulphated exo-polysaccharide	Marine Microalgae: *Gyrodinium* [73]	Inhibits replication by targeting viral internalization and incorporation steps	*In vivo* using HeLa cells, against encephalomyocarditis virus (EMCV). *In vitro* against influenza A virus.	[73,75]

432

433

434 Although genetic engineering of algae has grown in gigantic leaps, analyze and develop

435 bioprocesses based on algae strains with improved traits for an efficient production of native or

436 recombinant products are still required.

437 Usually, it is better to improve the production of a native product of the microorganism rather than a

438 recombinant one, this is achieved by the manipulation and modulation of existing metabolic pathways,

439 increasing the production and therefore the activity of certain enzymes along the desired pathway,

440 identifying the barriers that may affect our productivity and balancing every metabolic step ensuring

441 they are thermodynamically favorable for the desired production. By doing this, we ensure from the
442 beginning that the   product will be synthetized and focus on the genetical engineering of the existing
443 metabolic pathway to get high yields of the protein of interest.

444 As shown   in Figure 2, microalgae   constitute a viable alternative to common vaccines.
445 Additionally, it is a friendly road for developing, low-income countries as most of the production costs
446 could be avoided by the easiness of their culture, considering they have also been demonstrated to be
447 harmless for human consumption or even consumed before being considered as biorefineries for
448 antiviral molecules [73].

449

Microalgae

- RED: Chondrus, Gigartina, Hypnea & Euchema

-Brown: Laminaria, Ascophyllum, Macrocystis,

-Blue-green: Nostoc

-Brown sea weeds: Laminaria japonica, Eclkonia

Antiviral & antimicrobial molecules

-Carrageenan
-Alginates
-Galactans
-Fucans
-Nostoflan
-Calcium spirulan
-Naviculan
-A1 & A2 polysaccharide
-Laminarin
-p-KG03

Easier production

Less expensive

-Sunlight
-CO2
-Nutrients

Suitable for low-income countries

No need of complex installations
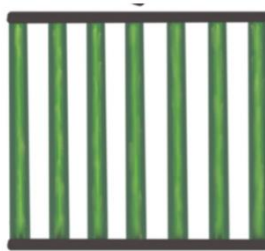
Photobioreactor

Administration vias: edible vaccines

Easier to administrate

No need of syringes, less waste

Cheaper transportation

Easier mainteinance & storage

Figure 2. Edible vaccines: microalgae as an alternative. Original image made with the online Biorender program.

7.1.1.1 Polymers and polysaccharides known mechanisms of action against viral infections

As mentioned in Table 2, there are several molecules suitable for uses against viral infections mainly. Most of those compounds have not been sufficiently studied enough to   elucidate their mechanisms

457 of action, since   turning them into potential antiviral treatments is considerably a recent idea. But in
458 the case of carrageenan, fucoidan and galactan, some mechanisms have been proposed as illustrated in
459 Figure 3. All three of these approaches coincide with the fact that carrageenan affects the binding of
460 the virus to the receptors in the hosts' cells.

461
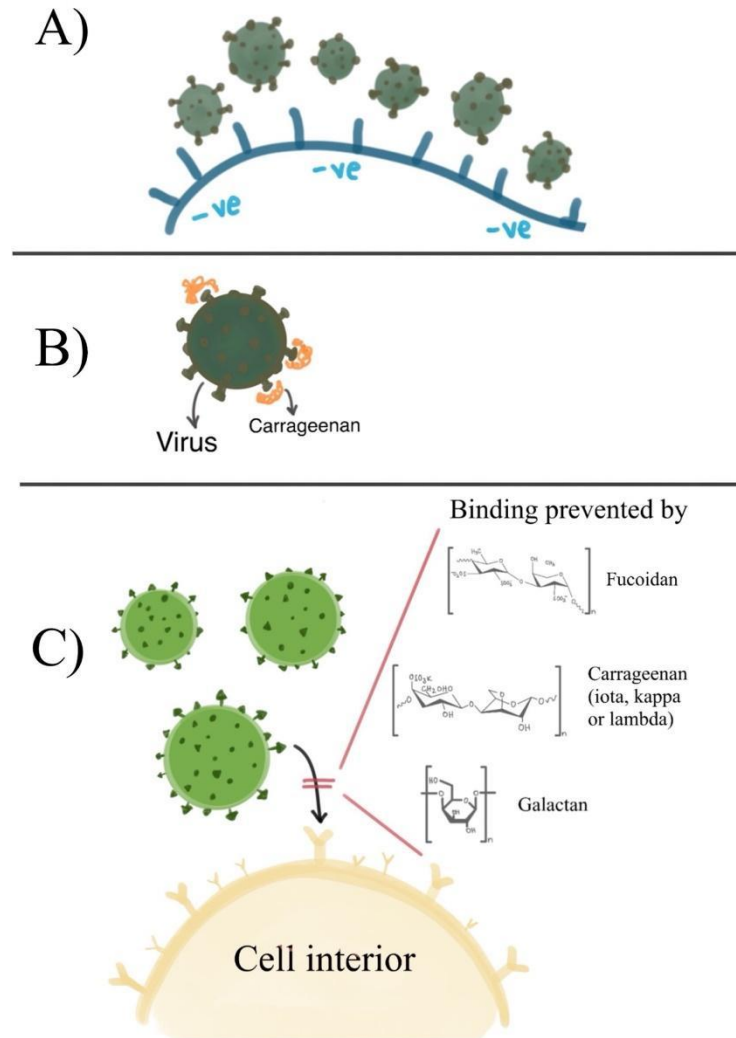
462
463 **Figure 3.** A) Negatively charged iota-carrageenan attracts and traps newly released positively charged
464 viruses. B) Inhibition mechanism of carrageenan by blocking viral attachment to host cells. C)
465 Carrageenan, Fucoidan and Galactan antiviral activity by receptor-virus binding inhibition.
466
467
468
469 Figure 3 A) is a proposed mechanism in which a negatively charged molecule of iota-carrageenan
470 interacts with newly released virus from the nasal epithelium, this mechanism contemplates common
471 cold virus as a target [83].

472   Although mechanisms B) and C) coincide with the approaches regarding the proposed mechanisms,
473   attachment of carrageenan to the virus in the same binding sites it would get attached to the cells, it is
474   important to mention that the type of carrageenan (iota, kappa or lambda) does not influence its activity
475   *in vitro*[74]. In the case of fucoidan and galactan, more precise mechanisms are yet to be elucidated
476   but it is also stipulated that they prevent viral binding [74,75,79,84].
477   Griffithsin (GRFT) is a lectin produced by red macroalgae *Griffthsia* sp. that has been proven to have
478   antiviral activity against HIV, SARS-CoV-1 and MERS-CoV S. Its mechanism of action is associated
479   to the formation of homodimeric complexes displaying three carbohydrate-binding domains, involving
480   specifically the tyrosine residues (Tyr 28, 68 and 110) per monomer that target mannose arrays in the
481   surface of pathogenic enveloped viruses. The specific mechanism of action of GRFT as adjuvant of
482   monoclonal antibodies (mAb) against HIV has been characterized and consists of the interaction of
483   GRFT and gp120, which leads to the display of CD-4 binding site, while the mAbs interact with the
484   CD-4 induced epitope. In the case o   f SARS-CoV-1, GRFT binds to the S glycoprotein and inhibits
485   viral entry depending on its concentration in the host. For MERS-CoV S, the mechanism of action
486   involves the interaction with mannoses from the MERS-CoV S envelope which affects their function
487   for the entry of the virus [72].
488   These approaches suggest a highly viable alternative to conventional high-cost vaccines, but there is
489   still a lot of studies to do towards the complete development of a suitable approach, considering the
490   complete characterization of the molecules, elucidation of their mechanisms of action and complete
491   downstream design.
492   Nonetheless, as these compounds prevent the infection once the virus is present in the organism perhaps
493   their use could be focused as an auxiliar treatment in case of an infection as well as a preventive agent.
494   The consumption of some algae may boost the immune response [85].
495

7.1.2   Transgenic microalgae-based edible vaccines

497   Another approach regarding the use of microalgae as vectors for oral vaccination is to modify them
498   genetically   to function as vectors for real epitopes. Transgenic microalgae can produce some
499   therapeutic proteins, as mentioned by Dyo & Purton (2018), mainly epitopes, such as CTB (Cholera
500   Toxin B) [86] have be expressed mainly in *C. reinhardtii*, nonetheless other proteins of pharmaceutical
501   interest have been produced using other microalgae like *Chlorella ellipsoidea,   Dunaliella salina,*
502   *Symbiodinium microadriaticum, Lotharella amoebiformis* and *Phaeodactylum tricornutum* [71]. As

503 shown in Table 3, there have been several approaches for antiviral proteins expressed in some of these
504 strains.

505 To produce these molecules, most of the research about genetic engineering of eukaryotic microalgae
506 is focused on chloroplast engineering, using the freshwater green alga *Chlamydomonas reinhardtii*. By
507 inserting transgenes into the small chloroplast genome instead of the nuclear the precision and
508 predictability of DNA surgery   increases greatly, thus the integration of the DNA into specific,
509 neutral loci inside the genome   homologously and stable, high-level, expression is achieved faster.
510 Regarding the folding steps of the proteins, disulfide bonds occur in the chloroplast quite easily and
511 fast, making them great for the folding of complex proteins such as epitopes, antibodies, antigens, and
512 other therapeutic proteins with more than one domain or containing subunits, as the chloroplast in
513 contrast with other living models as bacteria, contain a full range of chaperones, peptidylprolyl and
514 protein disulfide isomerases that contribute to the folding process [87,88].

515 It is also important to state that the chloroplast can   function as a safe sub   cellular site for hyper-
516 accumulation of recombinant protein without affecting   other cell mechanisms. This organelle can
517 accumulate and protect   a   considerably volume of proteins, that can reach as much as 60% of the
518 cell total volume [87–90].

519 Now, considering the viability of transgenic microalgae as vehicles for edible vaccines, they can be
520 effectively lyophilized and stored at room temperature for up to 20 months without losing antigenic
521 efficacy as the algae cell wall ensures a natural bioencapsulation to prevent the antigen degradation
522 by the GIT proteins   [91]. The green algae   cell wall   is composed of polysaccharides like
523 cellulose, some marine green algae also   have mannans and pectic substances   with cellulose [92].

524 These structural properties allow the algal cell wall to withstand harsh acidic conditions once
525 lyophilized, as demonstrated by Dressen et al, (2009), when assessing the stability of dried *C.*
526 *reinhardtii* they found it to be stable enough at pH 1.7, protecting intracellular contents from
527 proteolysis in the stomach and delaying their release until reaching the gut-associated lymphoid tissues
528 [93].

529 Depending on the approach of each research line, microalgae have been considered as microfactories,
530 serving as bioreactors for proteins with therapeutic potential, that once functional, are meant to be
531 extracted and purified for their use. This approach of biorefinery has several advantages, mostly the
532 cost-reduction of the process, even though the proteins still must be purified, the initial costs of highly

533 selective mediums used for traditional mammalian cells are discarded for easily scalable
534 photobioreactors.

535 The most attractive idea of using genetically engineered microalgae is their GRAS status, once the
536 protein of interest has been successfully synthesized by the microalgae, they could be consumed
537 without any kind of purification, opening the door to the edible vaccine biotechnology. Some of the
538 concerns about this approach rely on the difficulty of controlling the exact dosage of the protein that is
539 being consumed, as living factories, the amount of functional proteins synthesized by each microalgae
540 may vary slightly, which may cause an inconvenience for restrictive molecules that need to be carefully
541 dosed [85,94].

542 Other advantages of using microalgae as biofactories and delivery systems are their rapid
543 transformation rates, no need for growth regulators and their ability to properly fold complex proteins,
544 as the ones needed for vaccines. [66]

545
546 **Table 3.** Antiviral proteins expressed in different microalgae

Microalgae	Expressed protein	Targeted virus	Approach	Reference
Chlamydomonas reinhardtii	Foot-and-mouth disease virus VP1 protein fused with cholera toxin B	Virus VP1	For livestock, as edible vaccine	[86,95]
	E2 structural protein of classical swine fever virus (CSFV)	CSFV	For pigs, already tested in mice, the algae were used as bioreactors	[95,96]
	E7GGG, a mutated and attenuated form of the E7 oncoprotein of Human Papillomavirus	Papillomavirus	For humans, as bioreactors, requiring purification	[95,97]
	P24 (encoding the conical core subunit of HIV-1 viral particles)	HIV	For human use, production of viral and bacterial subunit protein, contemplating algae as bioreactors and a downstream process	[95,98]
	Envelope protein VP28 of white spot syndrome virus, a pathogen of crustaceans	White spot syndrome virus	For crustaceans, as an edible vaccine	[94,99]
	Haemagglutinin (HA) of avian influenza virus H5	Avian influenza Virus H5	For chickens, as an edible vaccine	[94,100]

	Large single-chain (lsc) antibody against glycoprotein D of herpes simplex virus	Herpes simplex virus	Human monoclonal antibodies for therapeutic use	[94,101]
Schizochytrium sp.	Hemagglutinin protein derived from A/ Puerto Rico/8/34 (H1N1) influenza	H1N1 influenza	For humans, using the algae as bioreactor, with the purified protein tested in mice.	[95,102]
	Subunits: viral (glycoprotein 1, GP1, from Zaire ebolavirus) and bacterial (the B subunit of Escherichia coli heat-labile enterotoxin, LTB)	Ebola virus	For human use, using algae as bioreactor, contemplating future purification	[95,103]
	B subunit of the heat labile *Escherichia coli* enterotoxin with 3 epitopes from the Zika virus envelope protein	Zika virus	For human use, proposed as an edible vaccine	[95,104]
Dunaliella salina	hepatitis B surface antigen (HBsAg)	Hepatitis B	For humans, using the microalgae as bioreactor, considering future purification	[95,105]
	VP28 envelope protein	White spot syndrome virus ART springer	For shrimps, as an edible vaccine	[106]
Chlorella pyrenoidosa	VP2 antigen of the Infectious bursal disease	IBD virus	For poultry, as edible vaccines	[95,107]

547

548   **7.2 Yeast-based edible vaccines**

549   Using yeast cells is a novel approach to broaden the edible vaccine portfolio. Yeasts have several

550   advantages that make them an appropriate choice for vaccine manufacturing, such as non-toxic nature,

551   simple and safe growing methods, simple genetic engineering, low production costs, and high cell

552   density in fermentation processes [108], [109]. Moreover, yeast display (YD) or yeast surface display

553   (YSD), a protein engineering tool, has been widely used to develop oral vaccines [110,111]. In fact,

554   two patented edible vaccines have been produced in yeast [112]. VisionTech International L. patented

555   a YSD system using S. cerevisiae to prevent White Spot Syndrome Virus (WSSV) infections in shrimps

556   [113]. This discovery could also be applied in preventing and treating diseases in humans. The

557   company Asahi Glass Ltd. also patented an edible vaccine. The inventors did not use YSD, instead

they took advantage of the avirulent fission yeast *Schizosaccharomyces pombe* to produce a prophylactic oral vaccine against human papilloma virus type 16 (HPV16) [114]. Besides those examples, several studies have used YSD in edible vaccines for animal diseases [111], but it is advancing as several studies and clinical trials are currently being developed [91].

For instance, the therapeutic vaccine GS-4774 for Hepatitis B (HBV) treatment is a recombinant heat-killed whole-yeast platform [115]. The Phase II clinical trial has already been published (registration number: NCT01943799). Besides, the vaccine GI-5005 is under clinical trials for Hepatitis C virus treatment [87]. This vaccine is also a heat-killed whole-yeast cell (registration number: NCT00124215). Although the mentioned vaccines are not edible, these studies are an example of the benefits of yeast-based vaccines and represent an important step in the development of whole-cell vaccines, which have the potential to be administered orally instead of the traditional parenteral route of administration [65].

Several biopharmaceuticals and vaccines produced in yeast hosts have been already approved by the FDA and EMA [116–118]. While these products are not edible, they confirm the advantages of using yeast cells for biopharmaceutical applications. In fact, no microalgae-based biopharmaceutical (either edible or parenteral) has been approved for commercial production and just a few have been tested in animals [87]. Moreover, the technology transfer to develop yeast-based vaccines is feasible, as several low- and middle-income countries have experience with fermentation processes [119]. Another advantage of whole-yeast oral vaccines is the simplified purification process because cellular lysis or protein purification are not needed, whereas the conventional production of recombinant antigens in yeast required lysis e.g., the Hepatitis B vaccine produced in *Saccharomyces cerevisiae* [120]. The immune response can also be improved using yeast cells, as their cell wall components have natural adjuvant activity, making the recombinant antigen more immunogenic [121].

7.3   Bacterial based edible vaccines

Some invasive bacteria have been used as live bacterial vaccine vectors, to synthesize and deliver heterologous antigens as vaccines, targeting diseases such as cancer and AIDS. This alternative to conventional vaccines has a remarkable advantage, bacteria can express more than one antigen and are mass produced easily, the delivery method can be either oral or intranasal.

There are two DNA vaccine carriers, the non-pathogenic bacteria, and the attenuated pathogen bacteria. In the case of attenuated pathogen bacteria, there have been some studies considering some attenuated bacteria such as *Salmonella*, *Listeria*, *Yersinia*, *Shigella* and *Mycobacterium bovis* BCG which have

been suggested as vectors due to their capability of triggering a strong immune response, these strains have to be attenuated in order to delete their pathogenic components and ensure safety for the host, there is yet another downside, when attenuated their capability to induce humoral and cellular immune responses decreases considerably [122,123].

About non-pathogenic bacteria, they have a great advantage over the pathogenic ones, as they do not represent any risk of infection in immunocompromised hosts. Some of these non-pathogenic bacteria considered here are as follows: *Lactic Acid Bacteria (LAB)* as the DNA Vaccine Carrier*:*   belong to the GRAS category and are quite resistant to the acidic gastrointestinal environment, thus   can deliver the vaccine to be correctly absorbed. Some of LAB strains are great probiotic bacteria, they help with lactose digestion, increase immune response by inhibiting pathogens proliferation in the gastrointestinal system and aid the mucosal immune system by activating plasma cells, inducing secretion of immunoglobulin A (IgA) and migration of T cells. *Lactococcus lactis* is the most studied LAB and a great option for DNA vaccines as delivery method because several genetic tool methods have been engineered specially for this strain. When consumed, this bacterium travels to the gut region and does not colonize it [124] and can deliver the DNA plasmid to host cells as shown by Guimarães et al. (2006) [125], Tao et al. (2011) [126], Yagnaric et al. (2016) [127] and Mancha-Agresi (2017) [128]. The most remarkable study so far is by Chatel et al. (2008), in which the transference of the DNA plasmid by *L. lactis* to *in vivo* murine epithelial cells was confirmed and the exogenous protein was expressed successfully by the mammalian cells [129].

Listeria monocytogenes is a Gram-positive bacterium that invades different cell types as epithelial, mucosal, macrophages, hepatocytes, DCs and epithelial cells in the blood-brain barrier. This great invasive ability triggers a high immune response and makes them ideal to target intestinal epithelium and therefore ensure the delivery of the DNA vaccine, using of course a non-pathogenic or attenuated, engineered strain [122].

Salmonella spp has been considered because its natural infection route is through the gastrointestinal pathway, making it easier for the vaccine to be delivered, nonetheless due to thar same pathogenicity two transgenic strains, mutant *S. thypi* and *S. typhimurium* have been engineered to suppress their production of aromatic substances and eliminate their ability to replicate. The most effective engineered strain so far was produced by Kong et al. (2012), an attenuated *Salmonella* with an hyper invasive phenotype that escapes the endosomes and reduces bacteria apoptosis therefore DNA is delivered into the host cell nucleus [130]. There have also been several other mutant strains developed to target other non-viral diseases such as cancer [122].

621 *Shigella spp.*, like *Listeria monocytogenes, Shigella* can evade endosomes, it also can also be retained
622 in the cytoplasmatic region and deliver DNA safely to the nucleus host cell. This microorganism can
623 invade lymphoid tissues and   generate high mucosal and systemic immune responses. Shata & Hone
624 (2001)[131] used a mutant *S. flexenery* to attenuate a HIV infection in a murine model, with a intranasal
625 delivery they achieved a similar immune response to the one induced by an intramuscular naked DNA
626 vaccine [122]. *Y. enterocolitica* is considered as a vector due to its ability to survive inside the host's
627 tissue for several days, along with the bacteria replication, the DNA vaccine replicates too and therefore
628 the amount of DNA vaccine increases [122]. Al-Mariri et al (2002) [132] also developed a DNA
629 vaccine against Brucella infection.

630 Lately, probiotics have been heavily considered for edible vaccines, as these organisms are responsible
631 for stimulating immune responses on their hosts and can modulate the risk or severity of certain
632 diseases, mainly in the gastrointestinal system, meaning they can either suppress unwanted immune
633 responses or stimulate the secretion of immunoglobulins. The activity of each bacteria and their
634 immunoregulatory mechanisms depends on the strain, underlying properties and ability to interact with
635 the native immune system. In this context, *Lactobacillus acidophilus* is considered the best candidate
636 for edible vaccines, while *Bacillus subtilis* has been proved to generate system-specific humoral and
637 mucosal immunity by oral administration, conferring protection against infectious diseases involving
638 for example, *Helicobacter* infection by promoting the production of specific IgA and systemic IgG. A
639 great advantage of *B. subtilis* is its stability, being able to remain viable at temperatures of 70ºC and
640 avoiding the need of cold chain systems for its handling   [65,133].

641
642 For these vaccines, the proposed delivery methods are   nasal, oral and   vaginal. The mucosal
643 delivery route is the most   studied,   it i   s non-invasive,   easier to control, and it induces
644 mucosal and systemic immune responses.   Nevertheless the oral route delivery is another
645 considerable option by not requiring any special skills, bacteria act as a protective capsule, keeping the
646 DNA vaccine material safely from the acidic environment of the stomach and the gut.   Nasal route
647 delivery   avoids the   unfavorable digestive system environment,   and also the potential
648 interference with enzymatic reactions, as well as inducing a higher immune response compared with
649 oral route [125].

650   **8. Conclusions**

651 The modern vaccines have many challenges and opportunities, and there are several important
652 approaches we must address: first, how do vaccines work; Second, what type of vaccine are we

653 reviewing, as each mechanism is different depending on the former; third, how are these vaccines being
654 produced; and finally, what else is out there that could give us a solution to current issues. Current
655 vaccine production platforms tend to require several specific and highly specialized facilities in order
656 to produce the required antibody, all of them also need highly qualified personnel and expensive down
657 streaming processes along with complex adjuvants and transportation protocols.

658 In this understanding, edible vaccines preceding from non-pathogenic sources such as microalgae,
659 bacteria and yeasts suppose great non-expensive alternatives, offering an easier production,
660 downstream, transportation and delivery methods compared to traditional vaccines. These
661 characteristics make   them accessible to low-income countries or remotely located towns where
662 cold-chain transportation is not possible. Nonetheless, there is still a large workload to develop, starting
663 with the complete elucidation of the mechanisms of action of the bioactive molecules present in yeasts
664 and microalgae, their antiviral capability, required doses and therefore delivery methods and
665 immunization strategies.

666

667 **Author Contributions**

668

669 KAG-G is the main author of the present work including tables and figures, SAP-B contributed with
670 the Yeast-based edible vaccines section, CC-Z, EMM-M, MAO-M, JES-H, RGA, HMNI, RP-S
671 participated in the preparation and revision of the manuscript. All authors listed have made substantial,
672 direct, and intellectual contribution to the work and approved it for publication.

673

689 **Conflict of Interest**

690 The authors declare that the research was conducted in the absence of any commercial or financial
691 relationships that could be construed as a potential conflict of interest.

692

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