

1 **Critical review on clinoptilolite safety and medical applications *in vivo***

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The published version can be found at: <https://doi.org/10.1016j.ejmech.2019.111833>
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22 **Abstract:**

23 Unique and outstanding physical and chemical properties of zeolite materials have made them
24 extremely useful in a variety of applications including agronomy, ecology, manufacturing and
25 industrial processes. Recently, a more specific application of one naturally occurring zeolite
26 material, clinoptilolite, has widely been studied in veterinary and human medicine. Due to a
27 number of positive effects on health, including detoxification properties, usage of clinoptilolite-
28 based products *in vivo* increased enormously. However, concerns have been raised in the
29 public of the safety of clinoptilolite materials for *in vivo* applications. Here, we review the
30 scientific literature on the health effects and safety in medical applications of different
31 clinoptilolite-based materials and propose some comprehensive, scientifically-based
32 hypotheses on possible biological mechanisms underlying observed effects on the health and
33 body homeostasis. We focus on clinoptilolite material safety and positive medical effects
34 related to detoxification, immune response and general health status.

35

36 **Keywords:** zeolite, clinoptilolite, toxicology, immunostimulation, antioxidant properties

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39 **Chemical properties and biological application of natural zeolite clinoptilolite**

40 Zeolites possess unique and outstanding physical and chemical properties. These
41 characteristics have made them very useful in a variety of applications including agronomy,
42 ecology, certain manufacturing, industrial processes, medicine and cosmetics. Recently,
43 application of a specific natural zeolite material, clinoptilolite, has been documented in
44 veterinary and human medicine. Subsequently, the market of clinoptilolite-based products for
45 use *in vivo* has constantly been growing (Figure 1) [1].

46 The name 'zeolite' originates from Greek words 'zeo', „to boil“, and 'litos', a stone. The current
47 nomenclature and classification of zeolite materials is given by the Structure Commission of
48 the International Zeolite Association that identifies each material based on their framework with
49 a three-letter mnemonic code; for instance, natural zeolite clinoptilolite is denoted as HEU [2].

50 By origin, zeolites can be natural, or synthetic materials. They are aluminosilicate minerals with
51 rigid anionic frameworks containing well defined channels and cavities. These cavities contain
52 metal cations which are exchangeable, or may also host neutral guest molecules that can also
53 be removed and replaced. Majority of natural zeolites are of volcanic origin and have a general
54 formula $M_{2/n}:Al_2O_3:xSiO_2:yH_2O$, where M stays for the extra-framework cation [3]. The
55 mineral structure is based on AlO_4 and SiO_4 tetrahedra, which can share 1, 2, or 3 oxygen
56 atoms, so there is a wide variety of possible structures, as the network is extended in three
57 dimensions. This unique structural feature is a basis for their well-known microporous
58 structure. Based on pore size and absorption properties, zeolites are among the most
59 important inorganic cation exchangers and are used in industrial applications for water and
60 waste water treatment, catalysis, nuclear waste, agriculture, animal feed additives, and in
61 biochemical applications [3].

62 The variety of zeolites' application is indeed a consequence of the porous structure: pores form
63 negatively charged channels and cavities, which are occupied with positively charged alkali
64 and alkali earth monovalent (*i.e.* Na^+ , K^+) and divalent (*i.e.* Ca^{2+}) ions, OH-groups or H_2O
65 molecules, which can be easily exchanged by other molecules and cations from the
66 surroundings (Figure 2). It is logical then, that the final Si/Al ratio in a zeolite determines ion
67 exchange capacity and attraction of cations that come to reside inside the pores and channels
68 [4,5].

69 Besides metal cations and water resident in zeolites' cavities and pores, other molecules and
70 cationic groups may be accommodated as well, such as, for instance, ammonia, and nitrate
71 ions, and all these are bound to different zeolites at different affinity levels (Journal of Water
72 Resource and Hydraulic Engineering 2014, 3 (4):74-80 Removal of Nitrate from Groundwater

73 by Using Natural Zeolite of Nizarneshwar Hills of Western India, R.W.Gaikwad, A.R.Warade).
74 For example, selectivity alignments of the zeolite clinoptilolite cation exchange have been
75 given as $Ba^{2+}>Cu^{2+}$, $Zn^{2+}>Cd^{2+}$, $Sr^{2+}>Co^{2+}$ by Blanchard et al. [6], as
76 $Pb^{2+}>Cd^{2+}>Cs^{+}>Cu^{2+}>Co^{2+}>Cr^{3+}>Zn^{2+}>Ni^{2+}>Hg^{2+}$ by Zamzow et al. [7], or as
77 $Co^{2+}>Cu^{2+}>Zn^{2+}>Mn^{2+}$ by Erdem et al. [8].

78 The mineral assemblies of the most common zeolite occurrences in nature are clinoptilolite-
79 and mordenite-containing tuffs, in which the zeolite clinoptilolite and mordenite content is high
80 (80% and over. It may come along with the aluminium phyllosilicate clay smactite (bentonite)
81 and accompanying phases present in lower percentages cristoballite, calcite, feldspar and
82 quartz. However, other types of zeolites (e.g. phillipsite, chabazite) and clay minerals may
83 dominate the mineral tuff assemblage and properties of such materials may vary in a widest
84 sense with respect to the final mineral content [9].

85 The widely tested zeolite suitable for medical applications *in vivo* is the clinoptilolite tuff, but
86 mordenite tuff was also studied by Selvam et al. (Natural Cuban zeolites for medical use and
87 their histamine binding capacity, T. Selvam, W. Schwieger, W. Dathe. Clay Minerals (2014) 49
88 (4): 501-512.). So far the word 'zeolite' has been used in the literature for different types of
89 zeolites, tuffs and clays. For example, both clinoptilolite and clay materials may be used for
90 ion-exchange reactions. Still, their structural properties and toxicology profiles may be different
91 (Environ Res. 2015 Apr;138:233-54. doi: 10.1016/j.envres.2014.12.024. Epub 2015 Feb 28.
92 Toxicological evaluation of clay minerals and derived nanocomposites: a review. Maisanaba
93 S, Pichardo S, Puerto M, Gutiérrez-Praena D, Cameán AM, Jos A). The structure of mineral
94 clays is, for instance, organized in layers (sheets), while clinoptilolite has tetrahedra arranged
95 so that they form large amounts of pore space in the crystals. Different physical-chemical
96 properties between clinoptilolite and clays, e.g. kaolinite were accordingly documented in the
97 literature [10,11,12,13,14,15 16, 17] For example, kaolinite structure may change during the ion-
98 exchange processes due to displacement of H^{+} ions, or due to swelling of the structure as a
99 consequence of Pb, Zn, or Cd cations absorption which is opposite to clinoptilolite constancy
100 during ion-exchange process [12].

101 Clinoptilolite shares a high structural similarity with the zeolite heulandite (they are
102 isostructural) and it is distinguished from heulandite by a higher silicon to aluminium ratio in
103 favour to silicon, where $Si / Al > 4.0$ and $(Na + K) > (Ca + Sr + Ba)$. The thermal behaviour of
104 clinoptilolite and heulandite is also different. The clinoptilolite structure is still not destroyed
105 after 12h of heating at $750^{\circ}C$, whereas the heulandite structure is destroyed after 12h at $450^{\circ}C$
106 [18]. This structural stability is an essential element for *in vivo* applications.

107 For instance, a synthetic material known as Zeolite A, used widely for ion-exchange in
108 industrial processes, has the framework composition with a high Al content and molar ratio of
109 Si / Al almost 1. This is indeed the highest aluminium content possible in tetrahedral
110 aluminosilicate frameworks [19]. In Zeolite A, the Al-framework is balanced by the maximum
111 number of cation exchange sites; it has high cation contents and superior exchange capacities.
112 However, it is not appropriate for *in vivo* applications, since similar to other low-silica zeolites,
113 zeolite A is unstable in acids. In contrast, zeolites with higher silica content, such as
114 clinoptilolite, are stable in acids [19].

115 We present a comprehensive review of clinoptilolite applications in veterinary and human
116 medicine. We consider all of the above clinoptilolite properties and propose its mechanisms of
117 action *in vivo* (summarized in Table 1) and propose some comprehensive, scientifically-based
118 hypotheses on possible biological mechanisms underlying observed effects on the health and
119 body homeostasis.

120

121 **Use of clinoptilolite in veterinary and human medicine**

122 Studies performed in the last decades showed a high potency of clinoptilolite in diverse medical
123 applications *in vitro* and *in vivo* [20]. A large number of documented positive clinoptilolite
124 medical effects were attributed to basic clinoptilolite material properties, in particular, to
125 reversible ion-exchange and adsorption capacity [5,20,21]. This central clinoptilolite
126 characteristic related to elimination of toxic agents and restoration of the body homeostasis
127 may be widely exploited in a number of medical applications.

128 For instance, a high affinity of clinoptilolite towards ammonia was proven in different systems
129 for elimination of ammonia from water [22,23,24]. This is why clinoptilolite has widely been
130 used for years in animal production as an additive to animal feed, or for removal of ammonia,
131 in animal manure [25]. This ammonia affinity is an interesting feature for medical applications
132 in humans as well. For example, detrimental roles of the end-products of protein fermentation,
133 such as ammonia, have been recognized on the colonic microbiota and epithelial health, in
134 particular on the colonocytes life span and function (Physiology of the Gastrointestinal Tract,
135 Volume 1, section 4, pp 744-749, Gut microbiome, ed. Hamid M. Said, sixth edition, Academic
136 press, London, 2018, Aliment Pharmacol Ther. 2016 Jan;43(2):181-96. doi:
137 10.1111/apt.13456. Epub 2015 Nov 2. Review article: insights into colonic protein
138 fermentation, its modulation and potential health implications. Yao CK, Muir JG, Gibson PR;
139 Colonic Protein Metabolism and Colorectal Cancer Curr. Issues Intest. Microbiol. (2000) 1(2):
140 51-58 R. Hughes, E.A.M. Magee, S. Bingham; Colorectal Carcinogenesis: A Cellular

141 Response to Sustained Risk Environment Kim Y. C. Fung, Cheng Cheng Ooi, Michelle H.
142 Zucker, Trevor Lockett, Desmond B. Williams, Leah J. Cosgrove, David L. Topping. Colorectal
143 Carcinogenesis: A Cellular Response to Sustained Risk Environment. 2018).

144 The excessive production of ammonia, but also of other gaseous products including CO₂ and
145 H₂S, may occur as a consequence of protein-rich, or imbalanced diets, or in diverse
146 pathogeneses where excessive protein fermentation occurs, including irritable bowel
147 syndrome, ulcerative colitis and colorectal carcinogenesis (Aliment Pharmacol Ther. 2016
148 Jan;43(2):181-96. doi: 10.1111/apt.13456. Epub 2015 Nov 2. Review article: insights into
149 colonic protein fermentation, its modulation and potential health implications. Yao CK, Muir JG,
150 Gibson PR; Colorectal Carcinogenesis: A Cellular Response to Sustained Risk Environment
151 Kim Y. C. Fung, Cheng Cheng Ooi, Michelle H. Zucker, Trevor Lockett, Desmond B. Williams,
152 Leah J. Cosgrove, David L. Topping. Colorectal Carcinogenesis: A Cellular Response to
153 Sustained Risk Environment. 2018). Clinoptilolite has a high affinity towards ammonium and
154 may prove useful in these cases as an adjuvant to the standard therapy [26]. From this
155 perspective, clinoptilolite was evaluated in a recent trial performed on aerobically trained
156 subjects [27]. In this study, endurance trained subjects were recruited and supplemented with
157 clinoptilolite/dolomite/maca based product (Panaceo Sport®). Athletes indeed, often report on
158 intestinal symptoms including nausea, stomach and intestinal cramps, vomiting and diarrhoea.
159 These symptoms may be a consequence of typical athletes' diets with high protein content, as
160 in such circumstances excessive protein fermentation may occur and is accompanied by
161 higher ammonia release in the intestine as well. These subjects also have increased intestinal
162 wall permeability. A well-known and complex relationship between exercise and oxidative
163 stress, depends on many diverse factors. For instance, regular moderate exercise increases
164 the resistance against oxidative stress, while acute and vigorous exercise can generate free
165 radicals in excess [63]. Consequences of exercise at exhaustion levels include increased
166 number of leukocytes due to damage of muscle fibres and connective tissue [64], as well as
167 elevated lipid-peroxidation marker MDA in the plasma [65]. It is therefore, not surprising that a
168 number of professional athletes present gastrointestinal symptoms which may end-up as
169 medical problems, infections and autoimmune disease [66,67]. Interestingly, supplementation
170 with Panaceo Sport® , positively influenced intestinal wall integrity, which was witnessed
171 through decreased concentrations of the tight junction modulator zonulin, a marker of
172 increased intestinal permeability [27].

173 Other studies on detoxification properties of clinoptilolite materials *in vivo* performed so far
174 were mainly on animals and they provide strong evidence on alleviating effects during
175 exposure to different toxicants upon clinoptilolite supplementation. For instance, prolonged
176 consumption of water with increased nitrate levels by dairy cattle is known to impair protein

177 metabolism and glucose utilization. In these cows, dietary administration of clinoptilolite
178 alleviated nitrate burden to the body and reduced negative systemic effects of nitrates [28].
179 Similarly, a dietary mixture containing 3% of a clinoptilolite-based product, showed to increase
180 nitrogen excretion in faeces and to lower nitrogen excretion in urine in growing pigs.
181 Importantly, no effects on protein retention values were observed and protein deposition was
182 not altered [29].

183 Moreover, clinoptilolite incorporated into the diet may be effective in fighting mycotoxins by
184 direct absorption. Affinity towards aflatoxins, zearalenone, ochratoxin and T2 toxin, was proven
185 *in vitro* in the presence of aminoacids and vitamins where the latter were not absorbed by
186 clinoptilolite material [31]. The specificity for aflatoxin M1 was also shown *in vivo* as well and
187 dietary administration of clinoptilolite, especially of the material with the smallest particle size,
188 at the rate of 200 g per cow per a day, effectively reduced milk aflatoxin M1 concentration in
189 dairy cattle [32].

190 It is important to note that supplementation with clinoptilolite in dairy cows may have additional
191 benefits, such as reduction of parturient paresis. A study by Katsoulos *et al.* for instance,
192 showed that clinoptilolite supplementation reduced its incidence and did not affected serum
193 concentrations of total calcium, phosphate, magnesium, potassium, and sodium [33]. This
194 veterinary application may be relevant for human health as well. Indeed, the demand for
195 healthier food products and balanced diets is being growingly recognized as a central paradigm
196 for preservation of the body's homeostasis and health. Moreover, it is widely known that
197 contamination of poultry by food-borne pathogens is considered among major problems in the
198 poultry industry. This is why antibiotics are standardly used in poultry meat production. Such
199 wide use of antibiotics in poultry, but also in production of other meat, has recently been
200 accepted as a major cause for development of antibiotic-resistant bacteria (Rustam I. Aminov,
201 Roderick I. Evolution and ecology of antibiotic resistance genes, Mackie FEMS Microbiol Lett
202 271 (2007) 147–16). New, natural possibilities for improvement of animal health in meat
203 production have therefore been widely discussed [34] and clinoptilolite may be a natural
204 alternative.

205 For instance, clinoptilolite has been tested as a possible supplementation to broilers feed as
206 an alternative to antibiotics for: (1) control of total flora at broiler farms, where clinoptilolite
207 supplementation showed a positive effect on of the total flora (Lipids Health Dis. 2012; 11: 35.
208 Effect of zeolite (clinoptilolite) as feed additive in Tunisian broilers on the total flora, meat
209 texture and the production of omega 3 polyunsaturated fatty acid. Zouhir Mallek, Imen Fendri,
210 Lamia Khannous, Amal Ben Hassena, Al Ibrahim Traore, Mohamed-Ali Ayadi, Radhouane
211 Gdoura), as well as on performance of production and organoleptic parameters, especially on

212 increase of omega 3 fatty acid levels in eggs [35]; (2) improvement of antioxidant capacity in
213 broilers where supplementation of clinoptilolite materials increased activities of glutathione
214 peroxidase, catalase, total superoxide dismutase and the total antioxidant capacity [36]; (3)
215 reduction of mycotoxin effects on broilers health, where the number of aflatoxin-affected
216 broilers, or the number of severe lesions in the liver of chickens, was reduced in the
217 clinoptilolite-supplemented group [37]. All these documented effects are due to clinoptilolite
218 capacity to adsorb harmful substances in the gastrointestinal tract that are not confined only
219 to micotoxins and ammonia, but include heavy metals and organic compounds as well.

220 Indeed, different studies showed that clinoptilolite materials provide direct detoxifying
221 performance *in vivo*. For instance, in lead-intoxicated mice, a clinoptilolite sorbent KLS-10-MA
222 decreased lead accumulation in the intestine by more than 70% [38,39]. Moreover, in rats
223 exposed to organophosphate poisoning, zeolite tuff containing 61% of clinoptilolite proved
224 efficient in restoration of cholinesterase activity in brain, liver, spleen, femoral muscle, heart,
225 stomach, duodenum, colon and erythrocytes of intoxicated animals [30]. It can generally be
226 stated that clinoptilolite loaded with potential toxicants in the intestine is then excreted along
227 with toxicants.

228 It seems that this detoxifying effect may have additional systemic effects. A role of clinoptilolite
229 has been recognized in medical applications, where usage in zootechnology and veterinary
230 medicine provided strong evidence on improvement of pets' fitness and efficiency in removal
231 of numerous harmful substances from the organism, including radioactive elements,
232 mycotoxins and poisons [40]. In addition, ethylenediaminetetraacetic acid (EDTA) and
233 clinoptilolite supplementation exerted a protective effect on the brain tissue of mice intoxicated
234 with lead by inducing antioxidant mechanisms and increasing activity levels of catalase,
235 superoxide dismutase, glutathione peroxidase, and glutathione [41]. Moreover, a study in
236 humans showed the ability of tribomechanically micronized clinoptilolite to decrease the
237 absorption of ingested ethanol by reducing blood alcohol levels at a dose of 5 g [42]. If the
238 clinoptilolite-containing product dosage is lower or if it is not administered at the time of alcohol
239 consumption, this effect may not be visible as shown by Gandy et al. (Clin Exp Gastroenterol.
240 2015; 8: 271–277. Potentiated clinoptilolite reduces signs and symptoms associated with
241 veisalgia. Justin John Gandy, Ilze Laurens, and Jacques Rene Snyman) where clinoptilolite
242 proved highly efficient in reduction of veisalgia symptoms and signs up to 40%–50%.

243 In addition, clinoptilolite has interesting antioxidant, haemostatic and anti-diarrheic properties
244 that may be exploited in human medicine, especially as adjuvants to standard therapies [1].
245 However, the number of clinical studies with clinoptilolite materials on humans is still low and

246 previously described immunomodulatory, anticancer and antioxidant effects of clinoptilolite *in*
247 *vivo* should be studied in more detail.

248 Even though the efficacy and potential of clinoptilolite materials in medicine seems high,
249 questions were raised on eventual clinoptilolite effects on physiologically relevant elements,
250 *i.e.* micronutrients and trace elements, or effects on important processes in the organism. The
251 results published thus far show that clinoptilolite does not affect the homeostasis of trace
252 elements and micronutrients and acts rather selectively on heavy-metals and toxicants. For
253 instance, clinoptilolite-treated dairy goats showed no changes in serum concentrations of fat-
254 soluble vitamins, macro-elements and trace elements, or activities of hepatic enzymes. In
255 addition, clinoptilolite supplementation improved milk fat percentage and milk hygiene [43]. No
256 effects of clinoptilolite on physiological mineral levels were observed in cows as well (Katsoulos
257 P.D., Roubies N., Panousis N., Arsenos G., Christaki E. , Karatzias H., Effects of long-term
258 dietary supplementation with clinoptilolite on incidence of parturient paresis and serum
259 concentrations of total calcium, phosphate, magnesium, potassium, and sodium in dairy cows.
260 *Am. J. Vet. Res.* 66 (2005) 2081-5.).

261

262 **Zeolites effects on oxidative stress and immune system**

263 In aerobic organisms, production of small quantities of reactive oxygen species (ROS),
264 including peroxides, superoxides, hydroxyl radicals, and singlet oxygen, occurs continuously
265 [44]. A controlled production of ROS is indeed essential to the body's homeostasis [45], while
266 excessive production of ROS is known to cause damage to the DNA, proteins and lipids [46].
267 Some ROS are produced endogenously, while others are derived exogenously, such as those
268 formed by ionizing radiation. The endogenous sources of ROS are the mitochondria,
269 cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation [47]. For
270 example, mitochondria produced ROS are the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide
271 (H_2O_2) and hydroxyl radical ($\bullet OH$). Other routes and factors may induce ROS in the organism
272 as well, such as ROS produced through the activity of xanthine oxidase, in reactions of
273 hypoxanthine to xanthine and xanthine to uric acid conversions, where molecular oxygen is
274 reduced into superoxide anion, followed by generation of hydrogen peroxide [48]. It is
275 understood that homeostasis in normal cells includes a balance between ROS production and
276 antioxidant defence activity. Indeed, antioxidant mechanisms in the human body that are the
277 main regulators of ROS levels are based on enzyme and non-enzyme systems. Enzyme
278 systems rely mainly on superoxide dismutase (SOD), catalase, peroxiredoxin (Prx),
279 thioredoxins (Trx) and glutathione (GSH) enzymes' activity, while non-enzymatic systems
280 comprise flavonoids, vitamin A, vitamin C, vitamin E and melatonin [49]. In addition to these

281 antioxidant systems inherent to the body, other exogenous antioxidants are important in
282 regulation of constant body's ROS homeostasis as well. For example, dietary compounds are
283 highly important for elimination of excessive ROS caused by external stimuli and include, for
284 instance, carotenoids, tocopherols, bioflavonoids, anthocyanins and phenolic acid [50]. When
285 ROS production exceeds antioxidant capacity, we usually perceive the process as "oxidative
286 stress" that leads to organic damage. Increased oxidative damage to cells and tissues and
287 modulation of the ROS regulated signalling pathways have recently been acknowledged in the
288 pathogenesis of a wide number of diseases, including obesity, atherosclerosis, heart failure,
289 uremic cardiomyopathy, kidney pathologies, hypertension, neurological disease and cancer
290 [51,52,53,54,55]. It should be noted that for a proper functioning of the body, antioxidant
291 defences, co-factors, or molecules that activate enzymes by binding to their catalytic sites are
292 also required. In case of antioxidant enzymes, these co-factors may include coenzyme Q10,
293 vitamins B1 and B2, carnitine, selenium and often transition metals Cu, Mn, Fe, and Zn [56].
294 Recently, a preliminary efficacy study performed on patients with dyslipidemia has also shown
295 a positive effect of clinoptilolite supplementation on lowering the total lipid count and LDL (low
296 density lipoproteins) which may also be indirectly correlated with its general antioxidative
297 effect (J Altern Complement Med. 2017 23(9):738-744. Clinoptilolite for Treatment of
298 Dyslipidemia: Preliminary Efficacy Study. Cutovic M, Lazovic M, Vukovic-Dejanovic V, Nikolic
299 D, Petronic-Markovic I, Cirovic D).

300
301 Due to a certain amount of pre-loaded elements, it is plausible to assume that clinoptilolite may
302 positively affect the body's metal homeostasis, including either the levels, or availability of
303 some physiological metal ions pre-loaded in the material, on signal pathways responsible for
304 production of endogenous antioxidant enzymes. This may partially underlie the observed
305 effects on the oxidative stress defence mechanisms, which are visible as activation or
306 restoration of activity and levels of natural antioxidant enzymes. Still, this effect should be
307 evaluated along with factors such as for example the applied daily dosage, health status or
308 lifestyle. For example, in the study of Lamprecht et al. [27], the daily dosage of 1.85 g
309 clinoptilolite material supplementation did show an effect on measured redox markers in blood
310 of healthy athletes. Further on, interesting effects of clinoptilolite supplementation were
311 documented in animals as well. In hepatectomized rats, for instance, common oxidative stress
312 markers are induced upon trauma including malondialdehyde (MDA) in the plasma and liver
313 tissue. When hepatectomized rats were supplemented with a micronized clinoptilolite
314 preparation, 'Froximun', MDA levels were significantly lower, while liver tissue antioxidant
315 mechanisms were strengthened, as witnessed by significantly higher activity of Cu-Zn SOD
316 and GSH [57]. Also, in chicken, daily supplementation with a natural clinoptilolite, or a modified
317 clinoptilolite, efficiently improved antioxidant capacity by increasing the antioxidant enzyme

318 activities in intestine mucosa, and decreasing the free radical NO content and inducible nitric
319 oxide synthase activity in the serum. Moreover, upon prolonged supplementation in chicken,
320 both tested clinoptilolite materials increased activities of glutathione peroxidase, catalase, total
321 SOD and total antioxidant capacity [58]. Similarly, in doxorubicin treated mice, micronized
322 clinoptilolite proved efficient in counteracting lipid peroxidation in the liver [59].

323

324 An interesting effect of clinoptilolite was observed in fluoride-intoxicated rats [60]. Fluoride is
325 neurotoxic upon penetration through the blood-brain barrier during gestation and post-
326 gestation periods. As a consequence of fluoride-intoxication, inhibition of antioxidant enzymes
327 occurred in pups along with lipid peroxidation. Upon supplementation of pups with clinoptilolite,
328 oxidative damage was restored and levels of GSH-Prx were substantially ameliorated in the
329 cerebral cortex and medulla oblongata. Similar results were however, observed in animals
330 supplemented with vitamins E and C as well [60]. In line with these results, it should also be
331 hypothesized that clinoptilolite might hold potential to combat acute fluoride-intoxication in
332 animals, as well as in humans. In the gastric juice, fluoride anions are converted into
333 hydrofluoride acid. Such weak hydrofluoride acid may form hydrogen bonds with the
334 clinoptilolite framework and be eliminated from the body in the stool.

335

336 It may be concluded however, that exact mechanisms of clinoptilolite effects on systemic
337 restoration of homeostasis and increased antioxidant capacity are still not fully understood, as
338 these effects are probably connected both to general detoxifying effects occurring in the
339 intestine, as well as to release of physiologically relevant cations from the clinoptilolite
340 framework during the ion exchange process, e.g. Ca, Mn, Zn, Mg, that are then readily
341 available to the organism and to the antioxidant mechanism. Similar indirect effects of
342 clinoptilolite on the antioxidant mechanisms in the body were also observed in different
343 pathologies and disease models. For instance, tribomechanically micronized zeolite increased
344 SOD activity in a transgenic mouse model of Alzheimer disease in the hippocampus and
345 cortex, while it concomitantly reduced A β (x-42) amyloid beta levels in the hippocampus [61].
346 Moreover, zinc-bearing clinoptilolite proved to exert a protective effect on performance and gut
347 health of broilers against *S. pullorum* infection and also to improve the SOD activity of ileal
348 mucosa and reduced MDA contents of jejunal and ileal mucosa [62].

349

350

351 It is also possible that antibacterial and antiviral effects of clinoptilolite might be in correlation
352 with immunomodulatory properties. For instance, in long term supplementation with
353 clinoptilolite, a decreased prevalence of *E. coli* carrying certain antimicrobial resistance and

354 virulence genes was documented [68]. An influence of natural clinoptilolite on *E. coli* was also
355 documented in another study on broilers *in vivo* [69]. In this study, a beneficial effect on
356 intestinal parameters was measured, which was hypothesized to be based on a direct effect
357 on the microbial population in the intestine. While the total count of *E. coli* was significantly
358 reduced, a rise of *Lactobacillus acidophilus* occurred in parallel [69]. Similarly, clinoptilolite
359 supplementation of Enterex®, approved by the Cuban Drug Quality Control Agency, showed
360 to be highly efficient in ameliorating diarrhoea symptoms in several clinical studies on humans
361 with acute diarrhoea of different aetiologies. Moreover, in cases where diarrhoea symptoms
362 were removed and the pathogenic agent was identified upon Enterex® treatment antibiotics
363 were additionally used to completely eliminate pathogenic bacteria from the intestinal lumen
364 [70]. Therefore, this observed antidiarrheal activity may be in correlation with Enterex® effect
365 on certain pathogenic bacteria count or microbiota status in general rather than with direct
366 antibacterial effect which would have to be confirmed by additional studies. Recently, a positive
367 effect of a potentiated clinoptilolite material (Absorbatox®) was also shown to reduce
368 symptoms associated with endoscopically negative gastroesophageal reflux disease and
369 nonsteroidal anti-inflammatory drug induced gastritis where it significantly prevented mucosal
370 erosion severity (Clin Exp Gastroenterol. 2014 7:215-20. Potentiated clinoptilolite: artificially
371 enhanced aluminosilicate reduces symptoms associated with endoscopically negative
372 gastroesophageal reflux disease and nonsteroidal anti-inflammatory drug induced
373 gastritis. Potgieter W, Samuels CS, Snyman JR).

374 Similarly, antiviral properties for clinoptilolite *in vitro* were shown on human adenovirus 5,
375 herpes simplex virus type 1 and human enteroviruses coxsackievirus B5 and echovirus 7 [71].
376 This effect may probably be attributed to a direct adhesion of viral particles on clinoptilolite *in*
377 *vitro* which then inhibits viral entrance in the cells and viral replication. Even though no *in vivo*
378 studies on clinoptilolite antiviral activity have been published thus far, positive
379 immunomodulatory effects were observed in patients treated for immunodeficiency disorders.
380 In a study performed by Ivkovic *et al.* [72], a significant increase in specific immunity cells
381 counts, B lymphocyte CD19+, T-helper cells CD4+ and activated T-lymphocytes HLA-DR+,
382 were observed in subjects treated with tribomechanically micronized clinoptilolite. This effect
383 was accompanied by significantly decreased natural immunity NK CD56+ cell counts. Again,
384 standard blood count parameters of patients remained within normal referent values [72].

385 A hypothesis for the observed clinoptilolite immunomodulatory effects may be the modulation
386 of the body defence mechanisms towards ROS. Indeed, ROS induces damage of cells and
387 tissues when inflammation is initiated as a mechanism for restoration of the body's
388 homeostasis. Any impairment of the host immune and inflammatory mechanisms in the long
389 term may cause other inflammatory disorders, e.g. chronic sinusitis, otitis media and

390 osteomyelitis, or microbial overgrowth syndromes, such as bacterial vaginosis, or inflammatory
391 bowel disorders. It is plausible therefore, to assume that such disorders have in common the
392 formation of biofilms due to impaired immunological reaction of the host organism [73].
393 Previous studies indeed, showed a link between antioxidative effect and the stimulation of the
394 immune system (Ann Clin Lab Sci. 2000 Apr;30(2):145-58. Review: Free radicals, antioxidants,
395 and the immune system. Knight JA; Nutr J. 2008; 7: 29. The role of antioxidant supplement in
396 immune system, neoplastic, and neurodegenerative disorders: a point of view for an
397 assessment of the risk/benefit profile Daria Brambilla, Cesare Mancuso, Mariagrazia Rita
398 Scuderi, Paolo Bosco, Giuseppina Cantarella, Laurence Lempereur, Giulia Di Benedetto,
399 Salvatore Pezzino, Renato Bernardini).

400 Clinoptilolite's positive immunomodulatory effects in similar conditions may be due to
401 interactions of clinoptilolite particles in the intestine with microfold cells (M-cells) (Figure 3). M-
402 cells are found in the gut-associated lymphoid tissue (GALT) of the Peyer's patches, a rich
403 lymphoid tissue that communicates with intestinal epithelial cells and the microbiome of the
404 intestine by diverse immunomodulation processes, as well as in the mucosa-associated
405 lymphoid tissue (MALT) of other parts of the gastrointestinal tract. These gastrointestinal cells
406 are known to initiate mucosal immunity responses on the apical membrane of the M-cells and
407 to allow transport of microbes and particles across the epithelial cell layer from the gut lumen
408 to the *lamina propria* where interactions with immune cells occur [74]. While evaluating
409 possible clinoptilolite immunomodulatory effects in the intestine, it should be emphasized that
410 M-cells can uptake nano- and submicro-particles, which can probably induce changes in redox
411 homeostasis in a cell [75]. These changes in the M-cells then affect the Payers patches as
412 well. It is important to note that M-cells apical and basolateral sides, which communicate with
413 Payers patches, are polarised [76] and one may hypothesize that due to this particular
414 phenotype, M-cells retain clinoptilolite particles or silica particles released from clinoptilolite
415 material (tuff) that do not enter the blood system (Clinoptilolite in Dextran Sulphate Sodium-
416 Induced Murine Colitis: Efficacy and Safety of a Microparticulate Preparation. Stéphane Nizet,
417 Eduardo Muñoz, Bernd L Fiebich, Peter M Abuja, Karl Kashofer, Kurt Zatloukal, Simone
418 Tangermann, Lukas Kenner, Cornelius Tschegg Dietmar Nagl, Laurenz Scheichl, DI Claudia
419 Meisslitzer-Ruppitsch Michael Freissmuth, Thomas Berger. Inflammatory Bowel Diseases
420 24(1), 2018, Pages 54–66) and act locally on this tissue. Contrary to M-cells, other cells in the
421 intestine cannot perform macropinocytosis and therefore cannot absorb negatively charged
422 clinoptilolite particles or silica particles released from clinoptilolite material (tuff) due to their
423 rich negatively charged glycoprotein-polysaccharide covering, glycocalix [77]. Some
424 probiotics' metabolites, e.g. from the lactic acid bacteria, exert the same activating function on
425 Payers patches as we suggest for clinoptilolite particles or silica particles released from

426 clinoptilolite material (tuff) and improve intestinal wall integrity [78]. Therefore, we propose that
427 this clinoptilolite-induced M-cells' communication with Payer's patches as similarly shown by
428 Pavelic *et al.* [79], either through particle intake or microbiota effect as recently shown in dogs
429 supplemented with the zeolite chabazite (Front Microbiol. 2016; 7: 1491. Modulation of the
430 Bifidobacterial Communities of the Dog Microbiota by Zeolite. Alberto Sabbioni, Chiara
431 Ferrario, Christian Milani, Leonardo Mancabelli, Enzo Riccardi, Francesco Di Ianni, Valentino
432 Beretti, Paola Superchi, Maria C. Ossiprandi), increases the immune response and in
433 particular, stimulates IgA producing B lymphocytes (plasma cells), a defensive mechanism of
434 the intestinal tract against pathogenic bacteria [80]. In a recent paper by Nizet *et al.* however,
435 (Clinoptilolite in Dextran Sulphate Sodium-Induced Murine Colitis: Efficacy and Safety of a
436 Microparticulate Preparation. Stéphane Nizet, Eduardo Muñoz, Bernd L Fiebich, Peter M
437 Abuja, Karl Kashofer, Kurt Zatloukal, Simone Tangermann, Lukas Kenner, Cornelius Tschegg
438 Dietmar Nagl, Laurenz Scheichl, DI Claudia Meisslitzer-Ruppitsch Michael Freissmuth,
439 Thomas Berger. Inflammatory Bowel Diseases 24(1), 2018, Pages 54–66), no clinoptilolite
440 particles were detected in the selected sections of the gut tissue. Even though the inspection
441 of a limited histopathological sections in this study cannot rule out the suggested hypothesis
442 on clinoptilolite particles or silica particles released from clinoptilolite material (tuff) in activation
443 of Payer patches, experimental analysis of the observed local immunomodulatory effect should
444 be done in more details. Indeed, microbiota – clinoptilolite interaction may also underlie this
445 mechanism as well as a role of IgA was already described in reduction of intestinal pro-
446 inflammatory signalling and bacterial epitope expression as part of the innate immune
447 mechanism that contributes to balancing antibodies negative impact on the microbiota status
448 [80]. Evidence was provided on the role of cross-talking between adaptive immune system and
449 gut microbiota by selective generation of immune responses to bacteria that consequently
450 stimulate the innate system and production of IgA. By this mechanism, the host can detect new
451 bacterial types and ignore previously encountered bacteria in the intestine [81]. This
452 immunomodulatory effect of clinoptilolite was speculated to be the so called 'silicate
453 superantigen' response. The superantigens generally encompass some bacterial exotoxins
454 and viral products with a potent non-specific immuno-stimulatory effect on large T-cells
455 fractions. This immunostimulation occurs upon simultaneous interaction of the superantigen
456 with MHC class II molecules and T-cell receptors. Superantigens bind to the variable V β region
457 of the T cell receptor, or to CD28 and do not follow the peptide-binding pattern. An incredibly
458 heterogeneous T cell clonal activation occurs upon binding and different cytokines are
459 produced massively [82]. The superantigen-activated T-lymphocytes provoke the cellular
460 immune response and also the humoral immune response, as postulated by Emmer *et al.* in
461 the multiple sclerosis pathogenesis as well [83]. Lymphocytes stimulation by silicates, which
462 also act as superantigens, was already shown for different silicate materials in the *in vitro*

463 conditions and this mechanism may underlie immunomodulation activity of clinoptilolite in the
464 intestine as well [84,85]. Even though the exact mechanisms remain elusive, one may
465 speculate that clinoptilolite silica or released silica acts as a superantigen that promotes
466 formation of IgA producing plasma cells, which is dependent on the presence of superantigen-
467 reactive T cells. A similar superantigen effect was already observed in Peyer's patches during
468 milk-borne mouse mammary tumour virus infection [86]. We cannot however, rule out some
469 other, unrecognized immunomodulatory effects of clinoptilolite due to a direct interaction with
470 human microbiome as well (Figure 3).

471 Majority of studies on clinoptilolite were done by use of different, so called, activated materials
472 to increase either the surface area, or to improve clinoptilolite general adsorption, or ion-
473 exchange capacity. Activation may be performed either by chemical treatment, e.g. with an
474 acid, by replacement of stabilizing cations, or by mechanical modifications by different
475 micronization methods, which may all increase the surface area, change ion-exchange
476 properties and adsorption capacity [87,88,89]. In the paper by Kraljevic Pavelic et al. (Kraljević
477 Pavelić, Sandra ; Micek, Vedran ; Filošević, Ana ; Gumbarević, Darko ; Žurga, Paula ; Bulog,
478 Aleksandar ; Orct, Tatjana ; Yamamoto, Yasuaki ; Preočanin, Tajana ; Plavec, Janez ; Peter,
479 Robert ; Petravić, Mladen ; Vikić-Topić, Dražen ; Pavelić, Krešimir Novel, oxygenated
480 clinoptilolite material efficiently removes aluminium from aluminium chloride-intoxicated rats in
481 vivo. Microporous and mesoporous materials (1387-1811) 249 (2017); 146-156), it was
482 specifically shown that different micronization methods change the clinoptilolite tuff properties
483 by affecting the surface area, pore size and silicon to aluminium ratio at the surface of the
484 material. Moreover, hydrochloric acid that is also present in the stomach may change
485 clinoptilolite physical chemical properties and was proven to enhance clinoptilolite ion-
486 exchange capacity for Cu^{2+} and Co^{2+} in a synthetic Cu-Co solution at concentrations relevant
487 for the stomach in vivo (0.1M) [90]. Still, the clinoptilolite ion-exchange effects *in vivo* are
488 complex and cannot be linearly explained as they are not affected only by the environmental
489 conditions (pH, temperature etc.) but also by the affinity properties of the material for other
490 cations as well. In a recent article, Turkish clinoptilolite was activated with hydrogen peroxide,
491 which acts as a weak acid, to improve Ni^{2+} ions removal from aqueous solutions [91]. The
492 authors show changes on the clinoptilolite surface upon activation that resulted in improved
493 Ni-ions absorption. This is important, as hydrogen peroxide dissociates into hydrogen ion H^+
494 and hydrogen peroxide radical ($\text{HO}_2\cdot$) and during the acid-activation process, H^+ ions are
495 brought to the negatively charged species on the material surface. As a consequence, de-
496 alumination of the surface occurs, which increases the Si/Al surface ratio and absorption
497 capacity for metal cations. This is a well-known process in industrial applications, while for the
498 *in vivo* applications, it may also hold certain relevance. *In vivo*, the acid concentrations of the

499 intestine are substantially lower than those used in industrial activation process. For instance,
500 gastric acid in the stomach contains hydrochloric acid (HCl) at 0.05 – 0.1 M. In such an
501 environment, a certain release of Al species from the clinoptilolite surface may well be
502 hypothesized even though aluminium from the clinoptilolite materials does not enter the blood,
503 or accumulates in the body as shown in athletes supplemented with zeolite-clinoptilolite
504 supplement [27] or healthy rats supplemented with different clinoptilolite materials (Kraljević
505 Pavelić, Sandra ; Micek, Vedran ; Filošević, Ana ; Gumbarević, Darko ; Žurga, Paula ; Bulog,
506 Aleksandar ; Orct, Tatjana ; Yamamoto, Yasuaki ; Preočanin, Tajana ; Plavec, Janez ; Peter,
507 Robert ; Petravić, Mladen ; Vikić-Topić, Dražen ; Pavelić, Krešimir Novel, oxygenated
508 clinoptilolite material efficiently removes aluminium from aluminium chloride-intoxicated rats in
509 vivo. Microporous and mesoporous materials (1387-1811) 249 (2017); 146-156) where
510 aluminium release into systemic circulation was observed only in rats supplemented with
511 synthetic zeolite A. The latter effect was attributed to the zeolite A lower stability in the acidic
512 pH of the intestine in comparison to clinoptilolite materials. In this study, authors also proved
513 that clinoptilolite materials were efficient in removal of aluminium from aluminium chloride-
514 intoxicated rats *in vivo*. These observations may be attributed to clinoptilolite stability, to low
515 bioavailability of Al species from water (around 0.1% to 0.4%), and immediate precipitation of
516 Al-species as non-soluble forms. Aluminium(III)-cation (Al^{3+}) has a generally strong affinity for
517 anions which promote its precipitation. The Al^{3+} in most situations seeks out complexing agents
518 with oxygen-atom donor sites, such as carboxylate or phosphate groups, e.g. from food in the
519 intestine. However, it should be noted that aqueous coordination chemistry of Al^{3+} , especially
520 in the living systems, is rather complex due to Al-complexes tendency to hydrolyse and form
521 polynuclear species, which vary according to the pH condition of the medium [92,93].
522 Interestingly, oral aluminium bioavailability is known to be increased by acidic pH, such as the
523 pH in the human intestine, but in case of clinoptilolite tuff, it may be decreased, as this is a
524 silicon-containing compound that releases certain amounts of water-soluble silica [20]. Data
525 has been provided on the ability of silicon-rich mineral water, or silicic acid to remove Al from
526 the human organism [94,95] and this Si and Al relation has been recognized as the main
527 evolutionary mechanism for fighting ecotoxicity of aluminium in living organisms. Water-soluble
528 silica forms may thus be acknowledged as important contributors to fighting aluminium
529 detrimental effects on human and animal health, especially nowadays when exposure to
530 bioavailable free aluminium cation is posing a serious problem due to industrial development
531 [96,97,98].

532 In addition, we hypothesize that previously observed data on antitumor properties of
533 clinoptilolite *in vitro* may be due to activation of clinoptilolite surface by acids. Even though in
534 majority of *in vitro* studies, the cells were grown in micronized clinoptilolite pre-treated growth

535 media, no ultracentrifugation was employed, which means that a colloid system containing
536 finest clinoptilolite particles was used for experiments (A clinoptilolite effect on cell media and
537 the consequent effects on tumor cells *in vitro*. Katic M, Bosnjak B, Gall-Troselj K, Dikic I,
538 Pavelic K. Front Biosci. 2006 May 1;11:1722-32; . Natural zeolite clinoptilolite: new adjuvant in
539 anticancer therapy. Pavelić K, Hadzija M, Bedrica L, Pavelić J, Dikić I, Katić M, Kralj M, Bosnar
540 MH, Kapitanović S, Poljak-Blazi M, Krizanac S, Stojković R, Jurin M, Subotić B, Colić M. J Mol
541 Med (Berl). 2001;78(12):708-20). For instance, it is well known that tumour cells have
542 increased hydrogen peroxide levels that regulate specific signalling pathways and hydrogen
543 peroxide may modify cysteine residues on antioxidative enzymes [99]. During modification,
544 enzymes are deactivated. Clinoptilolite can react with hydrogen peroxide (A novel Turkish
545 natural zeolite (clinoptilolite) treated with hydrogen peroxide for Ni²⁺ions removal from
546 aqueous solutions. Murat Canli, Yuksel Abali. Desalination and Water Treatment 57(15), 2016:
547 6925-6935), similar to other silica particles, and in such situations oxidative stress is induced
548 either through the breakdown of hydrogen peroxides to hydroxyl radicals, or through the
549 breakdown of hydrogen peroxides and production of the hydroperoxyl radicals [100].
550 Therefore, it is possible that the contact between clinoptilolite and tumour cells with increased
551 hydrogen peroxide concentrations induces formation of free radicals, so increases in oxidative
552 burden occur in tumour cells that consequently die. Tumor cells are susceptible to increased
553 oxidative stress and in our previous experiments this effect was not visible or was lower in
554 normal fibroblasts *in vitro* (A clinoptilolite effect on cell media and the consequent effects on
555 tumor cells *in vitro*. Katic M, Bosnjak B, Gall-Troselj K, Dikic I, Pavelic K. Front Biosci. 2006
556 May 1;11:1722-32). Also, it cannot be ruled out that some clinoptilolite particles enter into
557 tumour cells *in vitro*, as tumour cells are inherently depolarized [101] and can uptake particles
558 by endocytosis [102]. Recently, a new hypothesis has been suggested on the use of lipophilic
559 anions that target cancer cells due to their distinct electrical properties [103]. As clinoptilolite
560 particles are negatively charged polyanions, they might also target cancer cells and induce
561 additional oxidative stress upon entrance into the cytoplasm through hydrogen peroxide
562 activation, increased production of ROS and its consequent depletion within the cell. Depletion
563 of hydrogen peroxide and increased ROS production during hydrogen peroxide reaction with
564 clinoptilolite surface may change the redox status of the cell, e.g. through inhibition of the
565 transcription factor Nrf2. Indeed, in previous *in vitro* experiments on tumour cells, clinoptilolite
566 antitumour effects were attributed to modulation of the epidermal growth factor receptor (EGF-
567 R), protein kinase B (PKB)/Akt and nuclear factor kB (NfκB) signalling that are interconnected
568 with ROS and activity of Nrf2 [104,105]. This might be highly relevant for survival of cancer
569 cells as Nrf2 bears a proliferative role. In tumour cells, Nrf2 is usually activated by ROS-
570 induced oncogenes, such as KRAS and c-MYC [106], and inhibition of its activity may
571 contribute to apoptosis of tumour cells and abrogated tumour growth [107].

572

573 **Clinoptilolite toxicology in animals and humans**

574 The basic structure of clinoptilolite is considered to be biologically neutral and non-toxic [
575 HANDBOOK OF ZEOLITE SCIENCE AND TECHNOLOGY. Editors Scott M. Auerbach,
576 Kathleen A. Carrado, Prabir K. Dutta, CRC Press, 2003, New York-Basel]. The European Food
577 Safety Authority (EFSA) recently released an expert opinion on safety of natural zeolite
578 clinoptilolite *in vivo* [109]. EFSA evaluated and proved zeolite-clinoptilolite non-toxicity for
579 animal feed at doses 10000 mg/kg. Oral consumption of this type of zeolite, due to its extreme
580 chemical stability, in EFSA's opinion, does not represent a potential risk for *in vivo* applications
581 [109].

582 The first comprehensive acute, subchronic and chronic toxicology evaluation of a clinoptilolite
583 material *in vivo* was performed by Pavelic *et al.* [110]. In this preclinical toxicology study,
584 tribomechanically micronized clinoptilolite was evaluated at 'Ruđer Bošković' Institute in
585 Zagreb, Croatia, according to the standards and regulations required at the time by the
586 Organization for Economic Cooperation and Development (OECD). In that study, the effects
587 associated with increasing exposure times were analysed in three categories: 1) acute toxic
588 responses up to one month in mice and rats, 2) subchronic toxic responses up to three months
589 in mice and rats and 3) chronic toxic responses up to 1 year in rats and 6 months in mice.
590 Clinoptilolite was administered to the animals as a powder supplementing their usual diet.
591 Toxicity studies were approached by setting the "limit" test, which means that high doses of
592 the substance were applied during 15, or more days. Two doses were selected from the "limit"
593 test, 400 mg/mice/day (3.2 times higher than the dose specified by the regulatory agency) and
594 1000 mg/mice/day (8 times higher). Recalculated from human use, they were 10 times and 25
595 times higher than envisaged potential human exposure dosages (60g/75kg human body
596 weight and 150g/75 kg human body weight). The results showed that the "limit" test doses of
597 the substance did not cause death of mice. Therefore, "up and down" test on mice was
598 performed with doses ranging from 60-400 mg/mice/day. Again, no toxicity was observed.
599 Classical acute, subacute and chronic tests on rats and mice were performed as well. Oral (in
600 diet) administration to mice and rats showed no effects, or changes that could be correlated to
601 tribomechanically micronized clinoptilolite-supplementation. In addition, earlier in 1983, Pond
602 and Yen published a first study on the clinoptilolite effects on the reproduction and progeny
603 growth in rats with or without cadmium presence (Bull Environ Contam Toxicol. 1983
604 Dec;31(6):666-72. Reproduction and progeny growth in rats fed clinoptilolite in the presence
605 or absence of dietary cadmium. Pond WG, Yen JT.). They have shown protective effects of
606 clinoptilolite on haematocrit and haemoglobin levels as well as on cadmium levels in the liver

607 of pigs fed with cadmium in the presence of clinoptilolite in comparison with animals fed only
608 with addition of cadmium to the diet.

609 Similarly, in another study performed by the European Union Cosmetic Ingredient Review
610 Expert Panel, natural clinoptilolite showed no effects on female rat reproductive performance
611 and it proved non-genotoxic in the Ames bacterial test system [111]. Moreover, in an
612 independent study performed by Martin-Kleiner *et al.*, effects of tribomechanically micronized
613 clinoptilolite on the serum chemistry and haematopoiesis were evaluated in mice [112]. The
614 authors showed that ingestion of clinoptilolite was well tolerated and substantiated by
615 unchanged body mass in clinoptilolite supplemented mice. An increased level of potassium by
616 20% was detected in mice receiving the clinoptilolite-rich diet, while other changes in the serum
617 chemistry were not observed. Erythrocyte, haemoglobin and platelet levels in peripheral blood
618 were not affected by clinoptilolite supplementation either.

619 Also, Muck Seler and Pivac [113] studied effects of tribomechanically micronized and non-
620 micronized clinoptilolite materials on the serotonergic 5-hydroxytryptamine receptors 5-HT(1A)
621 and 5-HT(1B) in the brain of non-tumorous (control) and mammary carcinoma bearing female
622 mice. A reduced binding of 3[H]8-hydroxy-2-(di-n-propylamino)tetralin (3H-8-OH-DPAT) to 5-
623 HT(1A) receptors in mammary carcinoma bearing mice was normalized in animals
624 supplemented by tribomechanically micronized clinoptilolite. Also, administration of
625 clinoptilolite materials did not affect binding of 3H—8-OH-DPAT to studied receptors during
626 prolonged administration. The authors speculated that the observed effects in tumour-bearing
627 mice may be in correlation with electrolytes balance, or immune system response to
628 supplementation. A neuroprotective effect was also documented by Basha *et al.* [114]. Safety
629 of the material was also proven by Ivkovic *et al.* where no adverse reactions to
630 tribomechanically micronized clinoptilolite supplementation were observed in immunodeficient
631 patients [115].

632 Some concerns were raised in public on the possible lead leakage from the natural clinoptilolite
633 materials into the intestine. Still, extremely high affinity of clinoptilolite to lead has been
634 documented previously, where sorption of lead and cadmium (Cd) on natural clinoptilolite was
635 shown to be irreversible, or very slowly reversible [116] and in particular was shown to be high
636 in an acidic environment [117]. These results were obtained in very simple *in vitro* models that
637 may not adequately mimic human digestion. Further on, high capacity of zeolite lead
638 adsorption occurs in the pH range 3-11 [118] and leaching of lead from lead-preloaded
639 clinoptilolite occurs mainly in pH under 1, which is not relevant to conditions in the human body,
640 as shown by Petrakakis *et al.* [119]. The authors conducted the study according to the standard
641 procedures, Toxicity Characteristic Leaching Procedure/Environmental protection

642 agency/Resource Conservation and Recovery Act (TCLP/EPA/RCRA) (1311), EPA Methods
643 1310, 1320 and DIN 38414-S4, and provided evidence of the pH being the main factor affecting
644 Pb leaching from clinoptilolite. Interestingly, in the pH 3 and higher Pb, leakage was less than
645 1%, while at pH 1, leakage was observed up to 20% of the initial lead content. Furthermore,
646 the authors show that re-adsorption of Pb particles that leach from the solid material may occur
647 as well, and for lead this process occurred at pH 1.5 and 2. The Pb leaching percentage may,
648 in the authors' opinion, be generally correlated with an increasing initial load, but is not affected
649 by agitation rate, or particle size. Also, previously published results from trials on animals and
650 human subjects showed a strong clinoptilolite detoxifying effect and reduction of Pb content *in*
651 *vivo*. For instance, tissue lead concentrations in lead-intoxicated rats with or without
652 clinoptilolite supplementation clearly show that Pb concentrations were not increased in
653 animals fed with clinoptilolite and that the intoxication burden in animals can be even alleviated
654 by clinoptilolite supplementation [38,39,41]. Similarly, in the study by Fokas et al. (Animal Feed
655 Science and Technology 117(1–2) 2004, 121-129. Assessment of Pb retention coefficient and
656 nutrient utilization in growing pigs fed diets with added clinoptilolite. IP. Fokas, G. Zervas, K.
657 Fegeros, P. Zoiopoulos), clinoptilolite was added to the diet of growing pigs at 20 g/kg and no
658 significant increase of Pb concentration in blood and edible tissues was measured. In this study
659 however, Pb levels were not measured in the bones as the major storage compartment for
660 lead in the body and definite conclusions on eventual lead detoxification effects cannot be
661 therefore, derived from the presented data. Moreover, a clinical study comprising 22 human
662 subjects evaluated the effects of clinoptilolite treatment on chronic diseases which could be
663 traced back to heavy metal poisoning. During treatment with activated clinoptilolite from 7 to
664 30 days in total, both urine and blood serum were collected and tested for heavy metals and
665 electrolytes. In this study, the daily intake of activated clinoptilolite suspension was effective in
666 removal of toxic heavy metals from the body *via* the urine [108]. Another clinical study on
667 human subjects showed detoxifying effectiveness of clinoptilolite. A total of 102 heavy metal
668 contaminated men were investigated and decreased concentrations of harmful metals (Cd, Pb,
669 Cu, Cr, and Ni) were measured in their hair after a 30 days supplementation with clinoptilolite.
670 This decrease in harmful metal concentrations was a result of the clinoptilolite detoxification
671 function and probable restoration of the body mineral metabolism homeostasis [121].
672 Importantly, while in a classical detoxification process a great danger in removing the
673 physiologically important electrolytes from the serum exists, this has not been observed in
674 clinoptilolite trials both in humans and animals, where no substantial changes in physiologically
675 relevant trace elements, or vitamins were observed even after long-term administration
676 [108,122,123].

677 In conclusion, clinoptilolite materials tested in the scientific literature proved to be generally
678 safe for *in vivo* applications even though each material seem to retain its own physical-
679 chemical characteristics and exerts specific biological effects that cannot be readily
680 transferable to other materials. Different particle sizes, surface areas and cation compositions
681 may induce different biological effects and exert different levels of effectiveness. Biological
682 effects and toxicology data should therefore be carefully evaluated according to the type of
683 clinoptilolite material, or clinoptilolite-based preparations used in a particular study or
684 application. In this paper, presented literature on clinoptilolite effects *in vitro* and *in vivo* present
685 data for materials (tuffs) from different sources/continents, purity, chemical composition and
686 prepared for oral application by use of different milling processing methods. Moreover, the
687 research goals and experimental designs were different. This is why no generalization on the
688 mechanisms of action for clinoptilolite materials (tuffs) may be done at this point. Still,
689 presented studies deliver enough data to substantiate a generally safe profile and positive
690 medical effects for this types of materials, especially in the field of immunostimulation and
691 detoxification effects. In the future, it would be highly helpful to gather scientific data on direct
692 relationship between specific clinoptilolite material properties and sources with positive or
693 negative effects and mechanisms of action *in vivo*. This will fill in the current gaps in research
694 presented so far and as similarly suggested by Colella. Colella also emphasized the variability
695 and heterogeneity of the clinoptilolite material used in different applications and studies and
696 suggested to study in details applications and mechanisms of clinoptilolite materials in light of
697 known and well-established properties or behaviour (2011: Clay Minerals 46 (2): 295-309. A
698 critical reconsideration of biomedical and veterinary applications of natural zeolites. C. Colella).

699

700 **Conclusion**

701 In agreement with the scientific evidence presented in the literature so far, it can be generally
702 stated that clinoptilolite-based materials, including the so called activated materials, may be
703 regarded as safe for *in vivo* consumption. A variety of highly positive effects on animal and
704 human health were documented thus far for clinoptilolite-based materials. Due to clinoptilolite's
705 remarkable ion-exchange and adsorption properties and consequent detoxifying effects, it
706 proved useful in elimination of a variety of dangerous contaminants from the body and in
707 restoration of the impaired gut barrier. An indirect systemic detoxification effect attributed to
708 clinoptilolite-based material supplementation in the diet of both animals and humans was
709 documented in other organs as well, e.g. liver. However, the observed positive systemic
710 mechanisms are still not completely understood. We hypothesize that they may be at least
711 partially attributed to restoration of the human homeostasis due to local detoxification

712 properties within the intestine, release of soluble silica forms from the clinoptilolite tuff that
713 enter from the intestine into the blood, as well as to clinoptilolite's immunomodulatory effects.
714 The observed local immunomodulatory effects of clinoptilolite involve induction of immune
715 responses through the Peyer's patches and possible positive effects on microbial intestinal
716 populations through still unknown mechanisms. These local effects may have a systemic
717 'echo' on the whole immune status as well, as observed in some studies.

718 Finally, clinoptilolite's antioxidant effects and restoration of antioxidant defence mechanisms
719 may also be linked to the positive general systemic impact on health. However, conclusive
720 statements on the exact applications and benefits of clinoptilolite-based materials in humans
721 should be carefully investigated and analysed for each, specific clinoptilolite material, as the
722 mechanisms of action may have correlations with the specific material's physical and chemical
723 properties. Currently, different clinoptilolite-containing materials are used in medical
724 applications worldwide. These materials contain different percentages of clinoptilolite and
725 different compositions. Also, clinoptilolite-containing natural tuffs come with small quantities of
726 other trace elements and clinoptilolite is always pre-loaded with various cations. Some of the
727 alkaline ions contained in the crystal lattice, mainly Na⁺, Ca²⁺, Mg²⁺ and K⁺, may be readily
728 released during the ion-exchange process. Such clinoptilolite pre-loaded cation content and
729 percentage of clinoptilolite in the final composition might be relevant for studying medical
730 effects *in vivo*. While these parameters may not be that relevant for agricultural, or industrial
731 applications, veterinary and human applications would require a higher level of control via a
732 quality control system in the production, both of the raw material and the final products. For
733 example, a proper mining process with adequate cleaning, sieving, de-hydrating and pre-
734 milling processes, along with elemental and microbiological examination of the clinoptilolite
735 materials might be considered among essential requirements for ensuring purity and quality of
736 the final materials for *in vivo* consumption.

737

738 **Consent for publication**

739 Not applicable

740 **Availability of data and material**

741 Data sharing not applicable to this article as no datasets were generated or analysed during
742 the current study.

743 **Authors' contributions**

744 SKP generated the main idea and wrote the manuscript, generated and shaped presented
745 hypotheses, performed literature search and analysed, prepared figures and tables, discussed
746 and systematized all literature data; JSM prepared parts related to clinical trials, was involved
747 in discussion of clinical aspects and preparation of the table, DG performed literature search,
748 participated in writing of manuscript related to oxidative stress and immune system and
749 participated in shaping of hypothesis of zeolite molecular effects in vivo, AF performed
750 literature search on physical-chemical properties of clinoptilolite and wrote parts of the
751 manuscript related to clinoptilolite chemistry, NP performed a critical review of data and
752 literature, performed editing of the paper content and its final content, KP performed literature
753 search related to clinical aspects and toxicology, discussed clinical aspects of obtained results
754 and helped to draft the manuscript.

755 **Funding**

756 We greatly acknowledge the support of University of Rijeka research grants 13.11.1.1.11 and
757 13.11.1.2.01. NP was funded by the European Research Council (ERC) Starting Independent
758 Researcher Grant 278212, the Serbian Ministry of Education and Science Project III44006, the
759 Slovenian Research Agency (ARRS) project grant J1-8155 and the awards to establish the
760 Farr Institute of Health Informatics Research, London, from the Medical Research Council,
761 Arthritis Research UK, British Heart Foundation, Cancer Research UK, Chief Scientist Office,
762 Economic and Social Research Council, Engineering and Physical Sciences Research
763 Council, National Institute for Health Research, National Institute for Social Care and Health
764 Research, and Wellcome Trust (grant MR/K006584/1).

765 **Acknowledgements**

766 We greatly acknowledge JEOL Ltd., 1 Team, SM Application Department, Tokyo 196-8558
767 JAPAN and Mr. Yasuaki Yamamoto for providing us electronic microscope images of Zeolite
768 A and PMA clinoptilolite. We acknowledge the project "Research Infrastructure for Campus-
769 based Laboratories at University of Rijeka", co-financed by European Regional Development
770 Fund (ERDF).

771 **List of abbreviations**

772 HEU – clinoptilolite; Na – sodium; K – potassium; Ca – calcium; Ba – barium; Cu – copper; Zn
773 – zinc; Cd – cadmium; Sr – strontium; Co – cobalt; Pb – lead; Cs – caesium; Cr – chromium;
774 Ni – nickel; Hg – mercury; M – manganese; Al – aluminium; Si – Silicon; CO₂ – carbon dioxide;
775 H₂S – hydrogen sulphide; EDTA - ethylenediaminetetraacetic acid; Fe – iron; PMA –
776 micronized clinoptilolite material; ROS – reactive oxygen species; O₂^{•-} – superoxide anion;
777 H₂O₂ – hydrogen peroxide; •OH – hydroxyl radical; SOD – superoxide dismutase; Prx –

778 peroxiredoxin; Trx – thioredoxin; GSH – glutathione; MDA – malondialdehyde; AST – alanine
779 aminotransferase; ALT – aspartate aminotransferase; GGT – gamma-glutamyl transferase;
780 GALT – gut-associated lymphoid tissue; MALT – mucosa-associated lymphoid tissue; Al³⁺ –
781 Aluminium(III)-cation; EGF-R – epidermal growth factor receptor; (PKB)/Akt – protein kinase
782 B/Akt kinase; NfκB – nuclear factor κB; EFSA – The European Food Safety Authority; OECD
783 – Organization for Economic Cooperation and Development; 5-HT(1A) and 5-HT(1B) –
784 serotonergic 5-hydroxytryptamine receptors in the brain; 3H-8-OH-DPAT – 3[H]8-hydroxy-2-
785 (di-n-propylamino)tetralin; TCLP/EPA/RCRA – Toxicity Characteristic Leaching
786 Procedure/Environmental protection agency/Resource Conservation and Recovery Act.

787

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1107 **Tables**

1108 Table 1. Documented properties and effects of clinoptilolite relevant for biomedical applications
 1109 and effects in animals and humans .

Clinoptilolite properties	Clinoptilolite effects
Cation exchange capacity [1, 5, 21]	Detoxicant, mineral donor [Kraljevic Pavelic et al., 20, 108, 121]
Molecular sieve (size and shape selectivity) [1, 5]	Impact on the intestine status [27]
Selective adsorption of water [Adsorption of water by clinoptilolite and glauconite Kotova D.L., Artamonova M.N., Krysanova T.A., Novikova L.A., Belchinskaya L.I.. Сорбционные и хроматографические процессы 2016. 16 (3):390-95]	Immunomodulation [72, 79]
Removal of ammonia ions and uremic toxins (urea, uric acid, creatinine, p-cresol, indoxyl sulphate) [22-25, Iran International Zeolite Conference (IIZC'08) April 29 - May1, 2008, Tehran – Iran, IZC-08-239 Application of Zeolite in Biomedical Engineering: A Review. Sedigheh Jougehdoost, Sahebali Manafi]	Effect on pathogens and microbiota [68, 70, Prasai TP, Walsh KB, Bhattarai SP, Midmore DJ, Van TTH, Moore RJ, et al. (2016) Biochar, Bentonite and Zeolite Supplemented Feeding of Layer Chickens Alters Intestinal Microbiota and Reduces Campylobacter Load. PLoS ONE 11(4): e0154061]
Reversible binding of small molecules [1]	Enzyme mimetics, metalloenzyme mimicry [In: Biocatalysis and Biomimetics. Chapter 11, Norman Herron. Zeolite Catalysts as Enzyme Mimics. Toward Silicon-Based Life? pp 141–154, 1989, ACS Symposium Series, Vol. 392]
Biosensors [Oleksandr O Soldatkin, Margaryta K Shelyakina, Valentyna N Arkhypova, Esin Soy, Salih Kaan Kirdeciler, Berna Ozansoy Kasap,	Antitumour adjuvant [104, 105]

Florence Lagarde, Nicole Jaffrezic-Renault, Burcu Akata Kurç, Alexei P Soldatkin, Sergei V Dzyadevych. Nano- and micro-sized zeolites as a perspective material for potentiometric biosensors creation. *Nanoscale Research Letters* (2015) 10:59]

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1112 **Figure captions**

1113 Figure 1. The summary of the clinoptilolite effects on the human body and properties *in vivo*.
1114 Observed clinically relevant effects on organs and systems for different clinoptilolite materials
1115 *in vivo* are due to major clinoptilolite properties: detoxification, antioxidant effect, release of
1116 trace elements and positive influence on the microbiota status in the intestine. These effects
1117 were documented in animals and humans for clinoptilolite material used as supplementation
1118 to regular diet in a powdered form.

1119 Figure 2. Clinoptilolite structure: linked SiO₄ tetrahedra and pores with metal cations available
1120 for ion-exchange with environmental cations (e.g. caesium, Cs⁺) that are consequently trapped
1121 into the clinoptilolite structure. As naturally occurring clinoptilolite comes with pre-loaded
1122 cations (e.g. calcium, Ca²⁺), ion-exchange may occur depending on the ion-exchange capacity
1123 and cation affinity of the material, as well as on physical properties of the surrounding
1124 environment. In the herein presented example, Cs⁺ enters in the zeolite pores instead of Ca²⁺
1125 (adapted from <http://www.chemtube3d.com/solidstate/SS-Z-Clinoptilolite.htm> Creative
1126 Commons Attribution-Noncommercial-Share Alike 2.0 UK: England & Wales License).

1127 Figure 3. Proposed model of clinoptilolite positive immunomodulatory effect in the intestinal
1128 epithelium (denoted with red arrows) through interaction of clinoptilolite particles with microfold
1129 cells (M-cells). Clinoptilolite is denoted by 'C'. M-cells are hypothesized to transport luminal
1130 clinoptilolite particles across the epithelial barrier and present them to immunological cells (e.g.
1131 dendritic cells) in the lamina propria and the Peyer's patches. The latter are rich in T cells,
1132 macrophages, and clinoptilolite- activated IgA secreting B and plasma cells. The single layer
1133 of the intestinal epithelium is protected by mucus containing mucin glycoproteins where
1134 immunoglobulin A (IgA) and antimicrobial peptides prevent interaction of microbiota with the
1135 cell surface. Question marks (?) and blue arrows denote still unknown interactions of
1136 clinoptilolite with microbiota and microbiota with the lumen and epithelia.

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