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

Sandra Kraljević Pavelić[1]*, Jasmina Simović Medica[2], Darko Gumbarević[1], Ana Filošević, Nataša Pržulj[3], Kresimir Pavelic[1,4],

[1] University of Rijeka, Department of Biotechnology, Centre for High-throughput technologies, Radmile Matejčić 2, 51000 Rijeka, Croatia

[2] General Hospital Pula, Negrijeva 6/Zagrebačka 30, 5100 Pula, Croatia

[3] University College London, Computer Science Department, London, WC1E 6BT, UK

4 Juraj Dobrila University of Pula, Zagrebačka 30, 52100 Pula, Croatia

Correspondence:

Professor Sandra Kraljević Pavelić, University of Rijeka, Department of Biotechnology, Centre for High-throughput technologies, Radmile Matejčić 2, 51000 Rijeka, Croatia, sandrakp@biotech.uniri.hr,

E-mail addresses:

Jasmina Simović Medica jsmedica@obpula.hr
Darko Gumbarević darko.gumbarevic@biotech.uniri.hr
Ana Filošević ana.filosevic@biotech.uniri.hr
Kresimir Pavelic pavelic@biotech.uniri.hr, pavelic@unipu.hr
Nataša Pržulj natasa@cs.ucl.ac.uk

Abstract:

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

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

Chemical properties and biological application of natural zeolite clinoptilolite

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

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

By origin, zeolites can be natural, or synthetic materials. They are aluminosilicate minerals with rigid anionic frameworks containing well defined channels and cavities. These cavities contain metal cations which are exchangeable, or may also host neutral guest molecules that can also be removed and replaced. Majority of natural zeolites are of volcanic origin and have a general formula M2/n:Al2O3:xSiO2:yH2O, where M stays for the extra-framework cation [3]. The mineral structure is based on AlO_4 and SiO_4 tetrahedra, which can share 1, 2, or 3 oxygen atoms, so there is a wide variety of possible structures, as the network is extended in three dimensions. This unique structural feature is a basis for their well-known microporous structure. Based on pore size and absorption properties, zeolites are among the most important inorganic cation exchangers and are used in industrial applications for water and waste water treatment, catalysis, nuclear waste, agriculture, animal feed additives, and in biochemical applications [3].

The variety of zeolites' application is indeed a consequence of the porous structure: pores form negatively charged channels and cavities, which are occupied with positively charged alkali and alkali earth monovalent (*i.e.* Na^+, K^+) and divalent (*i.e.* Ca^{2+}) ions, OH-groups or H2O molecules, which can be easily exchanged by other molecules and cations from the surroundings (Figure 2). It is logical then, that the final Si/Al ratio in a zeolite determines ion exchange capacity and attraction of cations that come to reside inside the pores and channels [4,5].

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

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

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

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

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

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

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

Use of clinoptilolite in veterinary and human medicine

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

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

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

The excessive production of ammonia, but also of other gaseous products including CO_2 and H_2S, may occur as a consequence of protein-rich, or imbalanced diets, or in diverse pathogeneses where excessive protein fermentation occurs, including irritable bowel syndrome, ulcerative colitis and colorectal carcinogenesis (Aliment Pharmacol Ther. 2016 Jan;43(2):181-96. doi: 10.1111/apt.13456. Epub 2015 Nov 2. Review article: insights into colonic protein fermentation, its modulation and potential health implications. Yao CK, Muir JG, Gibson PR; Colorectal Carcinogenesis: A Cellular Response to Sustained Risk Environment Kim Y. C. Fung, Cheng Cheng Ooi, Michelle H. Zucker, Trevor Lockett, Desmond B. Williams, Leah J. Cosgrove, David L. Topping. Colorectal Carcinogenesis: A Cellular Response to Sustained Risk Environment. 2018). Clinoptilolite has a high affinity towards ammonium and may prove useful in these cases as an adjuvant to the standard therapy [26]. From this perspective, clinoptilolite was evaluated in a recent trial performed on aerobically trained subjects [27]. In this study, endurance trained subjects were recruited and supplemented with clinoptilolite/dolomite/maca based product (Panaceo Sport®). Athletes indeed, often report on intestinal symptoms including nausea, stomach and intestinal cramps, vomiting and diarrhoea. These symptoms may be a consequence of typical athletes' diets with high protein content, as in such circumstances excessive protein fermentation may occur and is accompanied by higher ammonia release in the intestine as well. These subjects also have increased intestinal wall permeability. A well-known and complex relationship between exercise and oxidative stress, depends on many diverse factors. For instance, regular moderate exercise increases the resistance against oxidative stress, while acute and vigorous exercise can generate free radicals in excess [63]. Consequences of exercise at exhaustion levels include increased number of leukocytes due to damage of muscle fibres and connective tissue [64], as well as elevated lipid-peroxidation marker MDA in the plasma [65]. It is therefore, not surprising that a number of professional athletes present gastrointestinal symptoms which may end-up as medical problems, infections and autoimmune disease [66,67]. Interestingly, supplementation with Panaceo Sport® , positively influenced intestinal wall integrity, which was witnessed through decreased concentrations of the tight junction modulator zonulin, a marker of increased intestinal permeability [27].

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

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

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

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

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

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

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

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

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

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

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

Zeolites effects on oxidative stress and immune system

In aerobic organisms, production of small quantities of reactive oxygen species (ROS), including peroxides, superoxides, hydroxyl radicals, and singlet oxygen, occurs continuously [44]. A controlled production of ROS is indeed essential to the body's homeostasis [45], while excessive production of ROS is known to cause damage to the DNA, proteins and lipids [46]. Some ROS are produced endogenously, while others are derived exogenously, such as those formed by ionizing radiation. The endogenous sources of ROS are the mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation [47]. For example, mitochondria produced ROS are the superoxide anion (O2•-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH). Other routes and factors may induce ROS in the organism as well, such as ROS produced through the activity of xanthine oxidase, in reactions of hypoxanthine to xanthine and xanthine to uric acid conversions, where molecular oxygen is reduced into superoxide anion, followed by generation of hydrogen peroxide [48]. It is understood that homeostasis in normal cells includes a balance between ROS production and antioxidant defence activity. Indeed, antioxidant mechanisms in the human body that are the main regulators of ROS levels are based on enzyme and non-enzyme systems. Enzyme systems rely mainly on superoxide dismutase (SOD), catalase, peroxiredoxin (Prx), thioredoxins (Trx) and glutathione (GSH) enzymes' activity, while non-enzymatic systems comprise flavonoids, vitamin A, vitamin C, vitamin E and melatonin [49]. In addition to these

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

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

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

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

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

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

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

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

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

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

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

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

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

Majority of studies on clinoptilolite were done by use of different, so called, activated materials to increase either the surface area, or to improve clinoptilolite general adsorption, or ion-exchange capacity. Activation may be performed either by chemical treatment, e.g. with an acid, by replacement of stabilizing cations, or by mechanical modifications by different micronization methods, which may all increase the surface area, change ion-exchange properties and adsorption capacity [87,88,89]. In the paper by Kraljevic Pavelic et al. (Kraljević Pavelić, Sandra ; Micek, Vedran ; Filošević, Ana ; Gumbarević, Darko ; Žurga, Paula ; Bulog, Aleksandar ; Orct, Tatjana ; Yamamoto, Yasuaki ; Preočanin, Tajana ; Plavec, Janez ; Peter, Robert ; Petravić, Mladen ; Vikić-Topić, Dražen ; Pavelić, Krešimir Novel, oxygenated clinoptilolite material efficiently removes aluminium from aluminium chloride-intoxicated rats in vivo. Microporous and mesoporous materials (1387-1811) 249 (2017); 146-156), it was specifically shown that different micronization methods change the clinoptilolite tuff properties by affecting the surface area, pore size and silicon to aluminium ratio at the surface of the material. Moreover, hydrochloric acid that is also present in the stomach may change clinoptilolite physical chemical properties and was proven to enhance clinoptilolite ion-exchange capacity for Cu^{2+} and Co^{2+} in a synthetic Cu-Co solution at concentrations relevant for the stomach in vivo (0.1M) [90]. Still, the clinoptilolite ion-exchange effects *in vivo* are complex and cannot be linearly explained as they are not affected only by the environmental conditions (pH, temperature etc.) but also by the affinity properties of the material for other cations as well. In a recent article, Turkish clinoptilolite was activated with hydrogen peroxide, which acts as a weak acid, to improve Ni^{2+} ions removal from aqueous solutions [91]. The authors show changes on the clinoptilolite surface upon activation that resulted in improved Ni-ions absorption. This is important, as hydrogen peroxide dissociates into hydrogen ion H^+ and hydrogen peroxide radical $(HO2\bullet)^-$ and during the acid-activation process, H^+ ions are brought to the negatively charged species on the material surface. As a consequence, de-alumination of the surface occurs, which increases the Si/Al surface ratio and absorption capacity for metal cations. This is a well-known process in industrial applications, while for the *in vivo* applications, it may also hold certain relevance. *In vivo,* the acid concentrations of the

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

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

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

Clinoptilolite toxicology in animals and humans

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

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

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

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

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

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

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

Conclusion

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

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

Finally, clinoptilolite's antioxidant effects and restoration of antioxidant defence mechanisms may also be linked to the positive general systemic impact on health. However, conclusive statements on the exact applications and benefits of clinoptilolite-based materials in humans should be carefully investigated and analysed for each, specific clinoptilolite material, as the mechanisms of action may have correlations with the specific material's physical and chemical properties. Currently, different clinoptilolite-containing materials are used in medical applications worldwide. These materials contain different percentages of clinoptilolite and different compositions. Also, clinoptilolite-containing natural tuffs come with small quantities of other trace elements and clinoptilolite is always pre-loaded with various cations. Some of the alkaline ions contained in the crystal lattice, mainly Na^{+}, Ca^{2+}, Mg^{2+} and K^{+}, may be readily released during the ion-exchange process. Such clinoptilolite pre-loaded cation content and percentage of clinoptilolite in the final composition might be relevant for studying medical effects *in vivo*. While these parameters may not be that relevant for agricultural, or industrial applications, veterinary and human applications would require a higher level of control via a quality control system in the production, both of the raw material and the final products. For example, a proper mining process with adequate cleaning, sieving, de-hydrating and pre-milling processes, along with elemental and microbiological examination of the clinoptilolite materials might be considered among essential requirements for ensuring purity and quality of the final materials for *in vivo* consumption.

Consent for publication

Not applicable

Availability of data and material

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

Authors' contributions

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

Funding

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

Acknowledgements

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

List of abbreviations

HEU – clinoptilolite; Na – sodium; K – potassium; Ca – calcium; Ba – barium; Cu – copper; Zn – zinc; Cd – cadmium; Sr – strontium; Co – cobalt; Pb – lead; Cs – caesium; Cr – chromium; Ni – nickel; Hg – mercury; M – manganese; Al – aluminium; Si – Silicon; CO_2 – carbon dioxide; H_2S – hydrogen sulphide; EDTA - ethylenediaminetetraacetic acid; Fe – iron; PMA – micronized clinoptilolite material; ROS – reactive oxygen species; O2•- – superoxide anion; H_2O_2 – hydrogen peroxide; •OH – hydroxyl radical; SOD – superoxide dismutase; Prx –

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

References

[1] Pavelić K. and Hadžija M., Medical Applications of Zeolites, in: Handbook Of Zeolites Science and Technology, Ed. Auerbach S.M., Carrado K. A., Dutta P. K., New York-Basel, CRC Press, 2003, pp.1143-1173.

[2] Baerlocher Ch., McCusker L.B. Olson D.H., Atlas of Zeolite Framework Types, 6th revised edition, 2007.

[3] Bogdanov B., Georgiev D., Angelova K., Yaneva K., Natural zeolites: Clinoptilolite Review, International Science conference "Economics and Society development on the Base of Knowledge", Stara Zagora, Bulgaria, 2009.

[4] Canli M., Abali Y.S., Bayca U., Removal of methelyne blue by natural and Ca and K-exchanged zeolite treated with hydrogen peroxide. Physicochem. Probl. Miner. Process. 49 (2013) 481− 96.

[5] Mumpton F.A., La roca magica: uses of natural zeolites in agriculture and industry. Proc. Natl. Acad. Sci. 96 (1999) 3463-70.

[6] Blanchard G., Maunaye M., Martin G., Removal of Heavy Metals from Waters by Means of Natural Zeolite, Water Res. 18 (1984) 1501–1507

[7] Zamzow M.J., Eichbaum B.R., Sandgren K.R., Shanks D.E., Removal of Heavy Metal and Other Cations from Wastewater Using Zeolites, Sep. Sci. Technol. 25 (1990) 1555–69.

[8] Erdem E., Karapinar N., Donat R., The removal of heavy metal cations by natural zeolites. J. Colloid Interface Sci. 280 (2004) 309-14.

[9] Cejka J. Zeolites and Ordered Mesoporous Materials: Progress and Prospects: The 1st FEZA School on Zeolites, Prague, Czech Republic, Gulf Professional Publishing, 2005.

[10] Svoboda D.L., Šulcová A.P., Characterization of kaolin dispersion using acoustic and electroacoustic spectroscopy, J. Min. Metall. B 44 (2008) 63-72.

[11] Bibi I., Mineralogy and Acid Neutralisation Mechanisms in Inland Acid Sulfate Environments. Doctoral Thesis, University of Sydney, Australia, 2012.

[12] Miranda-Trevino J.C., Coles C. A., Kaolinite properties, structure and influence of metal retention on pH. Appl. Clay. Sci. 23 (2003) 133–39.

[16] Dimowa L.T., Petrov S.L., Shivachev B.L., Natural and Zn exchanged clinoptilolite: in situ high temperature XRD study of structural behavior and cation positions, Bulg. Chem. Commun., 45 (2013) 466–73.

[17] Hecht K., A brief comment on the effect of natural clinoptilolite zeolite in the human body, Information material for doctors, naturopaths, therapists, pharmacists, chemists, and scientists, Adapted from: Karl Hecht, Elena Hecht-Savoley, 'Naturmineralien Regulation Gesundheit/Natural mineral health regulation', Schibri-Verlag, Berlin – Milow, 2005.

[18] Ghiara M. R., Petti C., Franco E., Lonis R., Luxoro S., and Gnazzo L., Occurrence of clinoptilolite and modernite in tertiary calc-alkaline pyroclastites from Sardinia (Italy). Clays and Clay Minerals, 47 (1999) 319-28.

[19] Payra P. and Dutta P. K., Zeolites: A Primer, in: S. M. Auerbach, A.Kathleen A. Carrado, P. K. Dutta, Handbook Of Zeolites Science and Technology editors, New York-Basel, CRC Press, 2003, pp.1-19.

[20] Jurkić L.M. , Cepanec I., Pavelić S.K. , Pavelić K. ,Biological and therapeutic effects of ortho-silicic acid and some ortho-silicic acid-releasing compounds: New perspectives for therapy, Nutr Metab (Lond) 10 (2013) 1-12.

[21] Pavelić K., Čolić M., Subotić B., Chapter 32-O-01 Biomedical application of zeolites. In: Studies in Surface Science and Catalysis, Zeolite and Mesporous Materials at the Dawn of the 21st Century, Proceedings of the 13th International Zeolite Conference, Volume 135, Elsevier, 2001.

[22] Zabochnichka-Światek M., Malinska K., Removal of ammonia by clinoptilolite Global NEST J., 12(3) 256-61.

[23] Demir A., Günay A., Debik E., Ammonium removal from aqueous solution by ion-exchange using packed bed natural zeolite, Water SA, 28 (2002) 329-35.

[24] Sprynskyy M. Lebedynets M., TerzykA.P., Kowalczyk P., Namiesnik J., Buszewski B., Ammonium sorption from aqueous solutions by the natural zeolite Transcarpathian clinoptilolite studied under dynamic conditions. J. Colloid Interface Sci. 28, (2005) 408–15.

[25] Handbook of Zeolite Science and Technology. Editors: Auerbach S.M., Carrado K.A., Dutta P.K., Marcel Dekker Inc., New York, 2003.

[26] Yao C.K., Muir J.G., Gibson P.R. Insights into colonic protein fermentation, its modulation and potential health implications. Aliment. Pharmacol. Ther., 43 (2016) 181–96.

[27] Lamprecht M., Bogner S., Steinbauer K., Schuetz B., Geilberger J.F., Leber B., Wagner B., Zinser E., Petek T., Wallner-Liebmann S., Oberwinkler T., Bachl N., Schippinger G., Effects of zeolite supplementation on parameters of intestinal barrier integrity, inflammation,

redoxbiology and performance in aerobically trained subjects, J. Int. Soc. Sports Nutr. 12 (2015) 40-51.

[28] Katsoulos P.D., Karatzia M.A., Polizopoulou Z., Florou-Paneri P., Karatzias H., Effects of prolonged consumption of water with elevated nitrate levels on certain metabolic parameters of dairy cattle and use of clinoptilolite for their amelioration, Environ. Sci. Pollut. Res. Int. 22 (2015):9119-26.

[29] Poulsen H.D., Oksbjerg N., Effects of dietary inclusion of a zeolite (Clinoptilolite) on performance and protein – metabolism of young growing pigs, Anim. Feed Sci. Techno. 53 (1995) 297-303.

[30] Mojzis J., Nistiar F., Kovac G., Mojzisova G., Preventive Effects of Zeolite in Swerer-Rat Intoxication with Vx-Substance, Vet. Med. 39 (1994) 443-49.

[31] Tomasevic-Canovic M., Dumic M., Vukicevic O., Duricic M., Jovanovic S., Adsorption effects of mineral adsorbent on the clinoptilolite basis, part 2: Adsorption behaviour in the presence of aminoacids and vitamins, Acta Veterinaria 46 (1996) 227-34.

[32] Katsoulos P.D., Karatzia M.A., Boscos C., Wolf P., Karatzias H., In-field evaluation of clinoptilolite feeding efficacy on the reduction of milk aflatoxin M1 concentration in dairy cattle, J. Anim. Sci. Technol., 58 (2016) 1-7.

[33] Katsoulos P.D., Roubies N., Panousis N., Arsenos G., Christaki E. , Karatzias H., Effects of long-term dietary supplementation with clinoptilolite on incidence of parturient paresis and serum concentrations of total calcium, phosphate, magnesium, potassium, and sodium in dairy cows. Am. J. Vet. Res. 66 (2005) 2081-5.

[34] Diaz-Sanchez S., D'Souza D., Biswas D., Hanning I., Botanical alternatives to antibiotics for use in organic poultry production. Poult. Sci. 94 (2015) 1419-30.

[35] Mallek Z., Fendri I., Khannous L., Ben Hassena A., Traore AI., Ayadi M.-A., Gdoura R., Effect of zeolite (clinoptilolite) as feed additive in Tunisian broilers on the total flora, meat texture and the production of omega 3 polyunsaturated fatty acid. Lipids Health Dis. 11 (2012) 35-42.

[36] Wu Y., Wu Q., Zhou Y., Ahmad H., Wang T., Effects of clinoptilolite on growth performance and antioxidant status in broilers., Biol. Trace Elem. Res., 155 (2013) 228-35.

[37] Ortatatli M., Oğuz H., Ameliorative effects of dietary clinoptilolite on pathological changes in broiler chickens during aflatoxicosis., Res. Vet. Sci. 71 (2001) 59-66.

[38] Beltcheva M. Metcheva R., Popov N., Teodorova S.E., Heredia-Rojas J.A., Rodríguez-de la Fuente A.O., Rodríguez-Flores L.E., Topashka-Ancheva M., Modified natural clinoptilolite detoxifies small mammal's organism loaded with lead, M. Biol. Trace Elem. Res., 147 (2012) 180-8.

[39] Beltcheva M. Metcheva R., Topashka-Ancheva M., Popov N., Teodorova S., Heredia-Rojas J.A., Rodríguez-de la Fuente A.O., Rodríguez-Flores L.E., Zeolites versus Lead Toxicity. J. Bioequiv. Availab. 7 (2015) 12-29.

[40] Laurino C. and Palmieri B., Zeolite: “the magic stone”; main nutritional, environmental, experimental and clinical fields of application. Nutr. Hosp. 32 (2015) 573-81.

[41] Basha M.P., Begum S., Mir B.A., Neuroprotective Actions of Clinoptilolite and Ethylenediaminetetraacetic Acid against lead-introduced toxicity in mice Mus musculus. Toxicol. Int. 20 (2013) 201-7.

[42] Federico A., Dallio M., Gravina A.G., Iannotta C., Romano M., Rossetti G., Somalvico F., Tuccillo C., Loguercio C., A pilot study on the ability of clinoptilolite to absorb ethanol in vivo in healthy drinkers: effect of gender. J. Physiol. Pharmacol., 66 (2015) 441-7.

[43] Katsoulos P.D., Zarogiannis S., Roubies N., Christodoulopoulos G., Effect of long-term dietary supplementation with clinoptilolite on performance and selected serum biochemical values in dairy goats, Am. J. Vet. Res., 70(3) (2009) 346-52.

[44] Hayyan M., Hashim M.A., AlNashef I.M., Superoxide Ion: Generation and Chemical Implications, Chem. Rev. 116 (2016) 3029–85.

[45] Covarrubias L. Function of reactive oxygen species during animal development: Passive or active? Dev. Biol., 320 (2008) 1–11.

[46] Gulam W., Ahsan H., Reactive oxygen species: role in the development of cancer and various chronic conditions, J. Carcinog. 5 (2006) 1-8.

[47] Inoue M. Sato E.F., Nishikawa M., Park A.M., Kira Y., Imada I., Utsumi K., Mitochondrial Generation of Reactive Oxygen Species and its Role in Aerobic Life, Curr. Med. Chem. 10 (2003) 2495-505.

[48] Valko M,, Izakovic M,, Mazur M,, Rhodes C.J., Telser J., Role of oxygen radicals in DNA damage and cancer incidence. Cell Biochem. 266 (2004) 37–56.

[49] Rahman K., Studies on free radicals, antioxidants, and co-factors, Clin. Interv. Aging, 2 (2007) 219–36.

[50] Smilin Bell Aseervatham G., Sivasudha T., Jeyadevi R., Arul Ananth D., Environmental factors and unhealthy lifestyle influence oxidative stress in humans—an overview, Environ. Sci. Pollut. Res. 20 (2013) 4356-69.

[51] Srikanthan K., Shapiro J.I., Sodhi K. The Role of Na/K-ATPase Signaling in Oxidative Stress Related to Obesity and Cardiovascular Disease Molecules. 21 (2016) 1-13.

[52] Miranda-Díaz AG, Pazarín-Villaseñor L, Yanowsky-Escatell FG, Andrade-Sierra J., Oxidative Stress in Diabetic Nephropathy with Early Chronic Kidney Disease, J. Diabetes Res. 2016 (2016) 1-7.

[53] Patel M. Targeting Oxidative Stress in Central Nervous System Disorders. Trends Pharmacol Sci. 37 (2016) 768-78.

[54] Ding C., Fan X., Wu G., Peroxiredoxin 1 - an antioxidant enzyme in cancer, J. Cell. Mol. Med., (2016), doi: 10.1111/jcmm.12955

[55] Chen X., Song M., Zhang B., Zhang Y., Reactive Oxygen Species Regulate T Cell Immune Response in the Tumor Microenvironment., Oxid. Med. Cell. Longev. 2016 (2016) 1580967.

927 [56] Khalid R. Studies on free radicals, antioxidants, and co-factors, Clin. Interv. Aging, 2
928 (2007) 219–36.

929 [57] Saribeyoglu K., Effects of clinoptilolite treatment on oxidative stress after partial
930 hepatectomy in rats, Asian J Surg. 34 (2011)153-57.

931 [58] Wu Y., Wu Q., Zhou Y., Ahmad H., Wang T. Effects of clinoptilolite on growth performance
932 and antioxidant status in broilers, Biol. Trace Elem. Res., 155 (2013) 228-35.

933 [59] Zarkovic N., Zarkovic K., Kralj M., Borovic S., Sabolovic S., Blazi M.P., Cipak A., Pavelic
934 K., Anticancer and antioxidative effects of micronized zeolite clinoptilolite. Anticancer Res., 23
935 (2003) 1589-95.

936 [60] Madhusudhan N., Basha P.M., Begum S, Ahmed F. Fluoride induced neuronal oxidative
937 stress and its amelioration by antioxidants in developing rats., Research report Fluoride., 42
938 (2009) 179–87.

939 [61] Montinaro M., Uberti D., Maccarinelli G., Bonini S.A., Ferrari-Toninelli G., Memo M.,
940 Dietary zeolite supplementation reduces oxidative damage and plaque generation in the brain
941 of an Alzheimer's disease mouse model, Life. Sci., 20 (2013) 903-10.

942 [62] Wang L.C., Protective effects of zinc-bearing clinoptilolite on broilers challenged with
943 Salmonella pullorum, Poult. Sci., 91 (2012) 1838-45.

944 [63] Boccatonda A., Tripaldi R., Davì G., Santilli F., Oxidative Stress Modulation Through
945 Habitual Physical Activity, Curr. Pharm. Des., 22 (2016) 3648-80.

946 [64] Morillas-Ruiz J.M. and Hernández-Sánchez P., Oxidative Stress and Antioxidant
947 Defenses Induced by Physical Exercise, Basic Principles and Clinical Significance of Oxidative
948 Stress, In: Basic Principles and Clinical Significance of Oxidative Stress, Ed. Sivakumar Joghi
949 Thatha Gowder, InTech, 2015.

950 [65] Pingitore A., Lima G.P., Mastorci F., Quinones A., Iervasi G., Vassalle C., Exercise and
951 Oxidative Stress: Potential Effects of Antioxidant Dietary Strategies in Sports, Nutrition, 3
952 (2015) 916-22.

953 [66] Waterman J.J., Kapur R., Upper gastrointestinal issues in athletes, Curr. Sports Med.
954 Rep., 11 (2012) 99-104.

955 [67] Oliveira E.P., Burini R.C., Jeukendrup A., Gastrointestinal Complaints During Exercise:
956 Prevalence Etiology, and Nutritional Recommendations, Sports Med., 44 (2014) 79-85.

957 [68] Jahanbakhsh S., Kabore K.P., Fravalo P., Letellier A., Fairbrother J.M., Impact of
958 medicated feed along with clay mineral supplementation on Escherichia coli resistance to
959 antimicrobial agents in pigs after weaning in field conditions, Res. Vet. Sci. 102 (2015) 72-79.

960 [69] Wu Q.J., Wang L.C., Zhou Y.M., Zhang J.F., Wang T., Effects of clinoptilolite and modified
961 clinoptilolite on the growth performance, intestinal microflora, and gut parameters of broiler,
962 Poult. Sci., 92 (2013) 684–92.

963 [70] Rodríguez-Fuentes G., Barrios M.A., Iraizoz A., Perdomo I., Cedré. B., Enterex: Anti-
964 diarrheic drug based on purified natural clinoptilolite, Zeolites, 19 (1997) 441–48.

965 [71] Grce M. and Pavelić K., Antiviral properties of clinoptilolite, Microporous and Mesoporous
966 Mater., 79 (2005) 165–69.

967 [72] Ivkovic S., Deutsch U., Silberbach A., Walraph E., Mannel M., Dietary supplementation
968 with an activated zeolite clinoptilolite in immunodeficiency: effects on the immune system. Adv.
969 Ther., 21 (2004) 135-47.

970 [73] Pincus S.Potential Role of Infections in Chronic Inflammatory Diseases. ASM News, 71
971 (2005) 529-35.

972 [74] Mabbott N.A., Donaldson D.S., Ohno H., Williams I.R., Mahajan A. Microfold (M) cells:
973 important immunosurveillance posts in the intestinal epithelium, Mucosal Immunol., 6 (2013)
974 666–77.

975 [75] Igarashi E., Nanomedicines and Nanoproducts: Applications, Disposition and Toxicology
976 in human body, CRC Press, 2015.

977 [76] Principles of Mucosal Immunology, Society for Mucosal Immunology, Ed. Smith P.D.,
978 MacDonald T.T., Blumberg R.S., Garland Science, New York, 2012.

979 [77] Egberts H.J., Koninkx J.F., van Dijk J.E., Mouwen J.M., Biological and pathobiological
980 aspects of the glycocalyx of the small intestinal epithelium. Vet Q., 6 (1984) 186-99.

981 [78] Sung H.K., Jeung W., Choi I.D., Jeong J.W., Lee D.E., Huh C.S., Kim G.B., Hong S.S.,
982 Shim J.J., Lee J.L., Sim J.H., Ahn Y.T., Lactic Acid Bacteria Improves Peyer's Patch Cell-
983 Mediated Immunoglobulin A and Tight-Junction Expression in a Destructed Gut Microbial
984 Environment, J. Microbiol. Biotechnol., 26 (2016) 1035-45**.**

985 [79] Pavelic K., Katic M., Sverko V., Marotti T., Bosnjak B., Balog T., Stojkovic R., Radacic M.,
986 Colic M., Poljak-Blazi M., Immunostimulatory effect of natural clinoptilolite as a possible
987 mechanism of its antimetastatic ability. J Cancer Res. Clin. Oncol. 128 (2002) 37-44.

988 [80] Round J.L., Mazmanian S.K., The gut microbiome shapes intestinal immune responses
989 during health and disease, Nat Rev Immunol. 9 (2009) 313–23.

990 [81] Peterson D.A., McNulty N.P., Guruge J.L., Gordon J.I., IgA Response to Symbiotic
991 Bacteria as a Mediator of Gut Homeostasis, Cell Host & Microbe, 2 (2007) 328–39.

992 [82] Proft T., Fraser J.D., Streptococcal Superantigens: Biological properties and potential role
993 in disease. In: Streptococcus pyogenes: Basic Biology to Clinical Manifestations [Internet]. Ed:
994 Ferretti J.J., Stevens D.L., Fischetti V.A., Oklahoma City University of Oklahoma Health
995 Sciences Center, 2016.

996 [83] Emmer A., Staege M.S., Kornhuber M.E. The retrovirus/superantigen hypothesis of
997 multiple sclerosis, Cell. Mol. Neurobiol. 34 (2014) 1087-96.

998 [84] Ueki A., Yamaguchi M., Ueki H., Watanabe Y., Ohsawa G., Kinugawa K., Kawakami Y.,
999 Hyodoh F., Polyclonal human T-cell activation by silicate *in vitro*. Immunology, 82 (1994) 332-
1000 5.

1001 [85] Aikoh T., Tomokuni A., Matsukii T., Hyodoh F., Ueki H., Otsuki T., Ueki A., Activation-
1002 induced cell death in human peripheral blood lymphocytes after stimulation with silicate in vitro,
1003 Int. J. Oncol., 12 (1998) 1355-59.

[86] Cabrera G., Vercelli C., Burzyn D., Piazzon I., Increases in IgA(+) B cells in Peyer's patches during milk-borne mouse mammary tumor virus infection are influenced by Toll-like receptor 4 and are completely dependent on the superantigen response, J. Gen. Virol., 91 (2010) 2814-20.

[87] Akimkhan A.M., Structural and Ion-Exchange Properties of Natural Zeolite, In: Ion Exchange Technologies, Ed. Ayben Kilislioğlu, InTech, 2012.

[88] Abdulkerim Y., Influence of acid activation on the ion-exchange properties of manisa-gordes clinoptilolite. Physicochem. Probl. Miner. Process., 48 (2012) 591−98.

[89] Canli M., Yuksel A., Ugur S. B., Removal of methylene blue by natural and Ca and K-exchanged zeolite treated with hydrogen peroxide, Physicochem. Probl. Miner. Process. 49 (2013) 481−96.

[90] MambaI B.B., NyembeI D.W., Mulaba-Bafubiandi A.F. The effect of conditioning with NaCl, KCl and HCl on the performance of natural clinoptilolite's removal efficiency of Cu2+ and Co2+ from Co/Cu synthetic solutions. Water SA, 36 (2010) 437-44.

[91] Çanli M.,Abali Y. A novel Turkish natural zeolite (clinoptilolite) treated with hydrogen peroxide for Ni2+ ions removal from aqueous solutions. Desalination and Water Treatment 57 (2016) 6925-35.

[92] Wesley R.H., Speciation of aluminum in biological systems, J. of Toxicol. Environ. Health 48 (1996) 543-68.

[93] Krewski D., Yokel R.A., Nieboer E., Borchelt D., Cohen J., Harry J., Kacew S., Lindsay J., Mahfouz A.M., Rondeau V., Human health risk assesement for aluminum, aluminium oxide, and aluminium hydroxyde., J. Toxicol. Environ. Health. B Crit. Rev. 10 (2009) 1–269.

[94] Davenward S., Bentham P., Wright J., Crome P., Job D., Polwart A., Exley C.l, Silicon-rich mineral water as a non-invasive test of the 'aluminum hypothesis' in Alzheimer's disease, Alzheimers Dis., 33 (2013) 423-30.

[95] Buffoli B., Foglio E., Borsani E., Exley C., Rezzani R., Rodella L.F., Silicic acid in drinking water prevents age-related alterations in the endothelium-dependent vascular relaxation modulating eNOS and AQP1 expression in experimental mice: an immunohistochemical study. Acta Histochem. 115 (2013) 418-24.

[96] Exley C., The toxicity of aluminium in humans, Morphologie, 100 (2016) 51-5.

[97] Beardmore J., Lopez X., Mujika J.I., Exley C. What is the mechanism of formation of hydroxyaluminosilicates?, Sci. Rep. 1 (2016) 1-8.

[98] Exley C., Darwin, natural selection and the biological essentiality of aluminium and silicon, Trends. Biochem. Sci., 34 (2009) 589–93.

[99] Lennicke C., Rahn J.,Lichtenfels R, A. Wessjohann, Seliger B., Hydrogen peroxide – production, fate and role in redox signaling of tumor cells, Cell. Commun. Signal., 13 (2015) 1-19.

[100] Rochette L., Vergely C.,Forgotten Radicals in Biology, Int. J. Biomed. Sci. 4 (2008) 255-59.

[101] Yang M., Brackenbury W.J., Membrane potential and cancer progression, Front. Physiol., 4 (2013) 1-10.

[102] Sincai M., Argherie D., Ganga D., Bica D., Comparative study of endocytosis between normal cells and those that had become tumor cells, LUCRĂRI ŞTIINŢIFICE MEDICINĂ VETERINARĂ, XL (2007) 610-612.

[103] Forrest M.D., Cancer cells have distinct electrical properties that predict a susceptibility to lipophilic anions; a new cancer drug paradigm. Cold Spring Harbor Laboratory, BioRxiVBeta (http://biorxiv.org/content/early/2015/12/24/035113), 2015

[104] Katic M., Bosnjak B., Gall-Troselj K., Dikic I., Pavelic K., A clinoptilolite effect on cell media and the consequent effects on tumor cells in vitro, Front. Biosci., 11 (2006) 1722-32.

[105] Pavelić K., Natural zeolite clinoptilolite: new adjuvant in anticancer therapy, J. Mol. Med. (Berl), 78 (2001) 708-20.

[106] DeNicola G.M., Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis, Nature, 475 (2011) 106–09.

[107] Ryoo I.G., Lee S.H., Kwak M.K., Redox Modulating NRF2: A Potential Mediator of Cancer Stem Cell Resistance, Oxid. Med. Cell. Longev., 2016 (2016) 1-14.

[108] Flowers J.L., Lonky S.A., Deitsch E.J., Clinical evidence supporting the use of an activated clinoptilolite suspension as an agent to increase urinary excretion of toxic heavy metals, Nutr. Diet. Suppl., 1 (2009) 11–18.

[109] Scientific Opinion on the safety and efficacy of clinoptilolite of sedimentary origin for all animal species, EFSA Journal, 11 (2013) 1-14.

[110] Pavelić K., Hadzija M., Bedrica L., Pavelić J., Dikić I., Katić M., Kralj M., Bosnar M.H., Kapitanović S., Poljak-Blazi M., Krizanac S., Stojković R., Jurin M., Subotić B., Colić M. Natural zeolite clinoptilolite: new adjuvant in anticancer therapy. J Mol Med (Berl). 78(2001) 708-20.

[111] Elmore A.R., Cosmetic Ingredient Review Expert Panel Final Report on the Safety Assessment of Aluminum Silicate, Calcium Silicate, Magnesium Aluminum Silicate, Magnesium Silicate, Magnesium Trisilicate, Sodium Magnesium Silicate, Zirconium Silicate, Attapulgite, Bentonite, Fuller's Earth, Hectorite, Kaolin, Lithium Magnesium Silicate, Lithium Magnesium Sodium Silicate, Montmorillonite, Pyrophyllite, and Zeolite, Int. J. Toxicol., 22 (2003) 37-102.

[112] Martin-Kleiner I. Flegar-Meštrić Z., Zadro R., Breljak D., Stanović Janda S., Stojković R., Marušić M., Radačić M., Boranić M., The effect of the zeolite clinoptilolite on serum chemistry and hematopoiesis in mice, Food Chem. Toxicol., 39 (2001) 717-27.

[113] Muck-Seler D., Pivac N., The effect of natural clinoptilolite on the serotonergic receptors in the brain of mice with mammary carcinoma, Life Sci., 73 (2003) 2059-69.

[114] Basha M.P., Begum S., Mir B.A., Neuroprotective action of clinoptilolite and ethylenediaminetetraacetic acid toxicity in mice Mus musculus, Toxicol Int., 20 (2013) 201-7.

[115] Ivkovic S., Deutsch U., Silberbach A., Walraph E., Mannel M., Dietary supplementation with the tribomechanically activated zeolite clinoptilolite in immunodeficiency: effects on the immune system, Adv. Ther., 21 (2004) 135-47.

[116] Hamidpour M., Kalbasi M., Afyuni M., Shariatmadari H., Holm P.E., Hansen H.C., Sorption hysteresis of Cd(II) and Pb(II) on natural zeolite and bentonite, J. Hazard. Mater., 181 (2010) 686-91.

[117] Perić J., Trgo M., Vukojević Medvidović N., Removal of zinc, copper and lead by natural zeolite — a comparison of adsorption isotherms, Water Res., 38 (2004)1893–99.

[118] Payne K.B., Abdel-Fattah T.M., Adsorption of Divalent Lead Ions by Zeolites and Activated Carbon: Effects of pH, Temperature, and Ionic Strength, J. Environ. Sci. Health. A — Tox. Hazard Subst. Environ. Eng., 39 (2004) 2275–91.

[119] Petrakakis Y., Mylona E., Georgantas D., Grigoropoluou H., Leaching of lead from clinoptilolite at acidic conditions, Global NEST Journal, 9 (2007) 207-13.

[120] Pond W.G., Krrok L.P., Ho H., Su D-R, Schoknecht P.A., Bone Density and Tissue Lead Accretion in Growing Rats Fed Low High Calcium with or without supplemental clinoptilolite, Bull. Environ. Contam. Toxicol., 57 (1996) 713-721.

[121] Zhakov Y.I. Clinical testing of the influence of Biologically Active Supplements (BAS) “Litovit” on the possibility of heavy metals excretion from the organism. Ministry of health of the Russian Federation, Chelyabinsk State Medical Academy.

[122] Katsoulos P.D., Roubies N., Panousis N., Karatzias H., Effects of long-term feeding dairy cows on a diet supplemented with clinoptilolite on certain serum trace elements, Biol. Trace Elem. Res., 108 (2005) 137-45.

[123] Papaioannou D.S., Kyriakis S.C., Papasteriadis A., Roumbies N., Yannakopoulos A, Alexopoulos C., Effect of in-feed inclusion of a natural zeolite (clinoptilolite) on certain vitamin, macro and trace element concentrations in the blood, liver and kidney tissues of sows, Res. Vet. Sci., 72 (2002) 61-8.

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 Joughehdoust, 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, metaloenzyme 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 microsized zeolites as a perspective material for potentiometric biosensors creation. Nanoscale Research Letters (2015) 10:59]

Drug carrier/delivery [Bonferoni MC, Gerri G: de' Gennaro M, Juliano C, Caramella C: Zn2+-exchanged clinoptilolite-rich rock as active carrier for antibiotics in anti-acne topical therapy: in-vitro characterization and preliminary formulation studies. Appl Clay Sci 2007; 36:95–102; Hayakawa K, Mouri Y, Maeda T, et al.: Surfactant modified zeolites as a drug-carrier and the release of chloroquin. Colloid Polym Sci 2000; 278:553–558]

Vaccine adjuvant [Garces JM. Observations on zeolite applications. In: Treacz MMJ, Marcus BK, Misher ME, Higgens JB, editors. Proceedings of the 12th International Conference on Zeolites. Warrendale: Materials Research Society; 1999:551–566]

1110

1111

Figure captions

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

Figure 2. Clinoptilolite structure: linked SiO_4 tetrahedra and pores with metal cations available for ion-exchange with environmental cations (e.g. caesium, Cs^+) that are consequently trapped into the clinoptilolite structure. As naturally occurring clinoptilolite comes with pre-loaded cations (e.g. calcium, Ca^{2+}), ion-exchange may occur depending on the ion-exchange capacity and cation affinity of the material, as well as on physical properties of the surrounding environment. In the herein presented example, Cs^+ enters in the zeolite pores instead of Ca^{2+} (adapted from http://www. chemtube3d.com/solidstate/SS-Z-Clinoptilolite.htm Creative Commons Attribution-Noncommercial-Share Alike 2.0 UK: England & Wales License).

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