In the Name of God, the Compassionate, the Merciful

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ON THE COVER

Hepatotoxicity. Histopathological features indicate lymphocytic inflammation, congestion, apoptosis, and lesser sinusoidal space in liver caused by diazinon (DZN), an organophosphorus pesticide administrated in 15 mg/kg doses in male rat. See page 37.

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The cryoprotective effects of erythritol on frozen-thawed ram sperm

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ABSTRACT

This study was conducted to evaluate the effect of replacing glycerol with erythritol on cryopreservation of ram spermatozoa. Semen samples (n=24) were collected from four rams in six times. In each session, the collected ejaculates (n=4) were pooled and split into 12 equal parts. The amount of 0.032 M glycerol (G32E0, equal to 3% glycerol), 0.016 M glycerol and 0.016 M erythritol (G16E16), 0.008 M glycerol and 0.024 M erythritol (G8E24), 0.032 M erythritol (G0E32), 0.054 M glycerol (G54E0, equal to 5% glycerol), 0.027 M glycerol and 0.027 M erythritol (G27E27), 0.013 M glycerol and 0.041 M erythritol (G13E41), 0.054 M erythritol (G0E54), 0.076 M glycerol (G76E0, equal to 7% glycerol), 0.038 M glycerol and 0.038 M erythritol (G38E38), 0.019 M glycerol and 0.057 M erythritol (G19E57) and 0.076 M erythritol (G0E76) were added. The diluted samples were frozen using standard protocol. After thawing, the samples were incubated at 37°C for 6 h. Results showed that progressive sperm motility and acrosome integrity were higher in G13E41 (18.85 % and 27.41 %, respectively) than treatments that contained only glycerol at 6 h ($p < 0.05$). At the level of 0.032 and 0.054 M cryoprotectant, the highest of total sperm motility was observed in G8E24 (19.16 %) and G13E41 (18.85 %) at 6 h, respectively ($p < 0.05$). Therefore, the quality of frozen-thawed ram spermatozoa can be improved by using the mixture of 0.013 M glycerol plus 0.041 M erythritol or 0.008 M glycerol plus 0.024 M erythritol.

Keywords

Cryopreservation, Polyol, Glycerol, Ram sperm

Abbreviations

G32E0: group treated with 0.032 M glycerol
G16E16: group treated with 0.016 M glycerol + 0.016 M erythritol
G8E24: group treated with 0.008 M glycerol + 0.024 M erythritol
G0E32: group treated with 0.032 M erythritol
G54E0: group treated with 0.054 M glycerol
G27E27: group treated with 0.027 M glycerol + 0.027 M erythritol
G13E41: group treated with 0.013 M glycerol + 0.041 M erythritol
G0E54: group treated with 0.054 M erythritol
G76E0: group treated with 0.076 M glycerol
G38E38: group treated with 0.038 M glycerol + 0.038 M erythritol
G19E57: group treated with 0.019 M glycerol + 0.057 M erythritol
G0E76: group treated with 0.076 M erythritol

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Introduction

Cryopreservation allows for the long-term storage of spermatozoa, which is highly advantageous in a number of reproductive fields. Also, it is vital for animal genetic maintenance and propagation. The semen of ram can be frozen for long-term storage, but when the fertility rate is acceptable, frozen-thawed spermatozoa should be inseminated using intrauterine insemination via the laparoscopic method [1]. Yet, sheep breeders would rather use the vaginal or intracervical artificial insemination, as simple and cheap methods, than laparoscopic intrauterine artificial insemination [2]. Therefore, it seems necessary to improve the freezing methods of ram spermatozoa.

Glycerol has been used commonly as a cryoprotectant in the freezing of diluted ram semen [3]. However, there is evidence that the presence of glycerol in the diluted semen depresses fertility in ovine [4]. Moreover, glycerol accelerates the acrosome reaction of ram spermatozoa [5, 6]. The negative effects of glycerol have led to studies on the replacement of glycerol with other cryoprotective agents [7, 8]. However, these studies did not come with satisfactory results.

Erythritol is a four carbon sugar alcohol and this polyol can be used as a cryoprotective agent [9, 10]. In the mammalian cell, erythritol is not metabolized to toxic metabolites, but, glycerol can produce toxic agents [11]. The aim of the present study was to evaluate the effect of replacing glycerol with erythritol on the process of cryopreservation of ram spermatozoa.

Results

Results showed that erythritol did not affect the membrane integrity, viability, total and progressive sperm motility, curvilinear velocity, average path velocity and straight line velocity at 0 and 3 h after thawing ($p > 0.05$).

At the level of 0.032 and 0.054 M cryoprotectant, the highest of total sperm motility was observed in G8E24 (29.64 % ± 1.41) and G13E41 (24.74 % ± 1.20) at 6 h, respectively (Fig 1, $p < 0.05$). There was no difference between treatment content of 0.076 M cryoprotectant on the total sperm motility at 6 h ($p > 0.05$). Progressive sperm motility was higher in G8E24 (19.16 % ± 1.18) and G13E41 (18.85 % ± 1.42) than G32E0 (11.1 % ± 0.56), G3E1 (11.92 % ± 1.09), G54E0 (11.04 % ± 0.44), G27E27 (11.09 % ± 0.29), G76E0 (12.80 % ± 1.68) and G0E76 (11.44 % ± 0.50) at 6 h (Fig 2, $p < 0.05$). Curvilinear velocity (Fig 3), average path velocity (Fig 4) and straight line velocity (Fig 5) was higher in G8E24 (50.65 ± 2.41, 31.07 ± 1.48, 30.22 ± 1.44, respectively) than treatments with content of 0.032 and 0.076 M cryprotectant at 6 h after thawing ($p < 0.05$)

There was no difference among G8E24 (22.45 % ± 0.58), G27E27 (22.92 % ± 1.96), G13E41 (23.42 % ±0.76) and G19E57 (22.57 % ± 0.39) on the sperm viability at 6 h (Fig 6, $p > 0.05$). Sperm viability was higher in these treatments than G32E0 (17.47 % ± 0.47) and G76E0 (17.22 % ± 0.74) at 6 h ($p < 0.05$).

Membrane integrity was higher in G13E41 (23.43 % ± 1.04) than G32E0 (18.08 % ± 0.83) at 6 h (Fig 7, $p < 0.05$) and there was no difference among other treatments ($p < 0.05$).

Acrosome integrity was higher in G13E41 than G8E24, G0E32, G0E54, G76E0, G19E57 and G0E76 at 0 h (Table 1, $p < 0.05$). Acrosome integrity was higher in G13E41 than G32E0, G0E32, G76E0 and G19E57 at 3 h ($p < 0.05$). Acrosome integrity was higher in

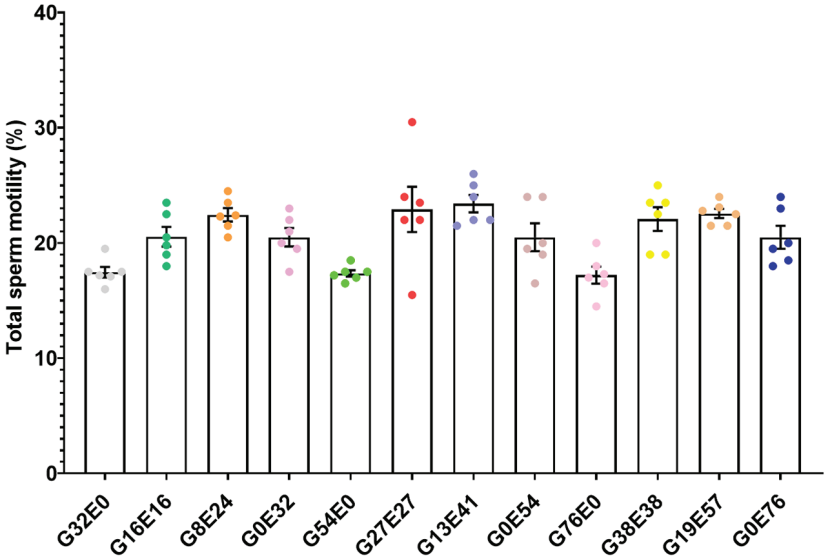

Figure 1
Effect of glycerol and erythritol on the total motility of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

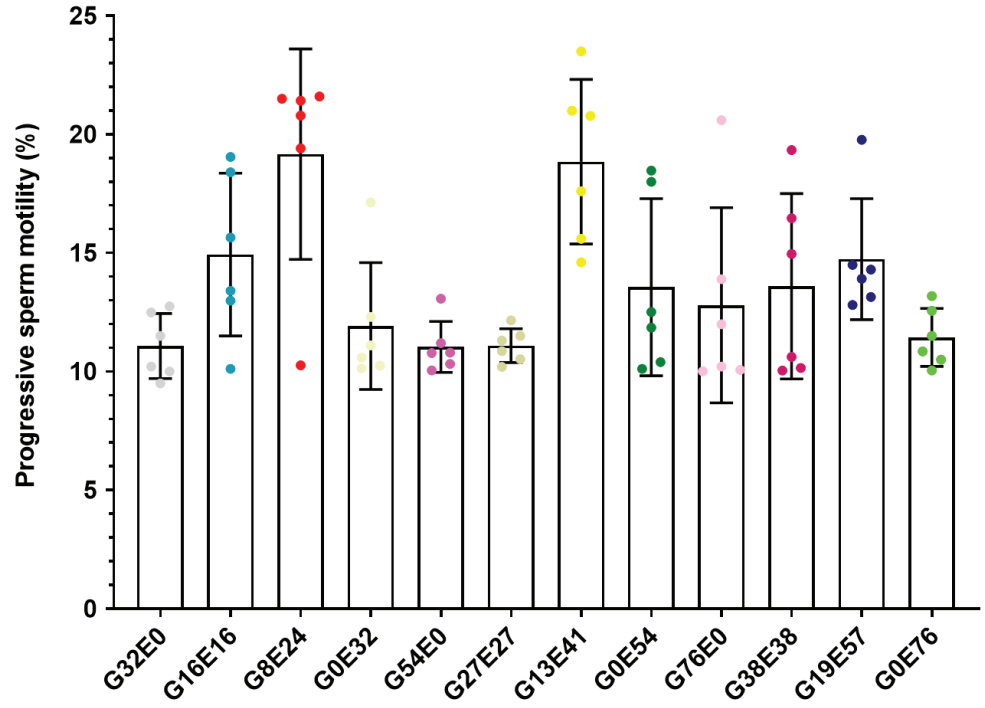

Figure 2
Effect of glycerol and erythritol on the progressive motility of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

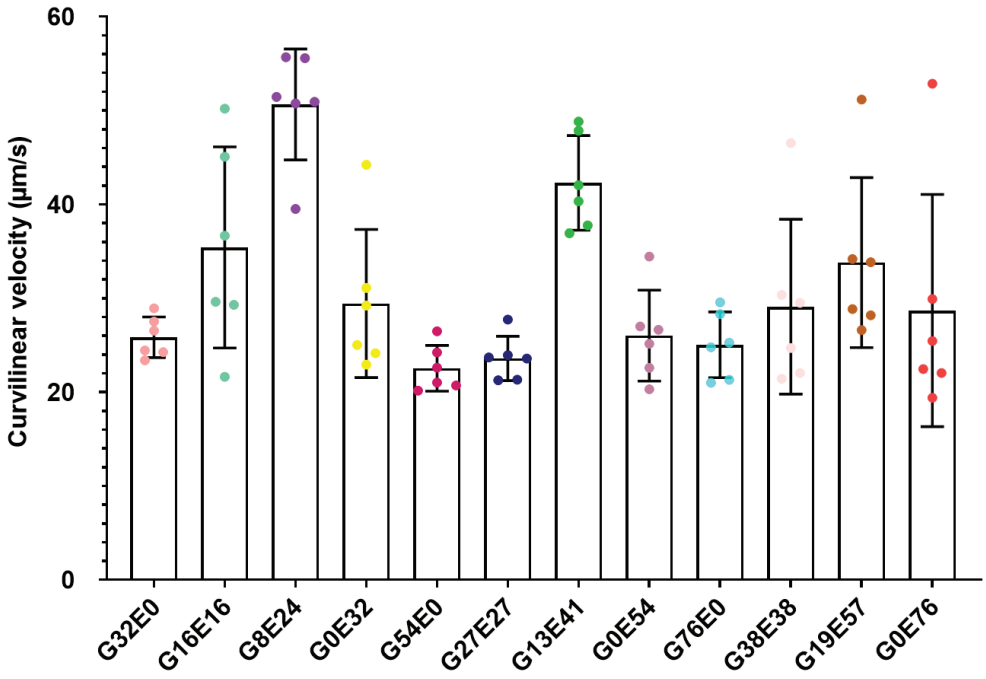

Figure 3
Effect of glycerol and erythritol on the curvilinear velocity of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

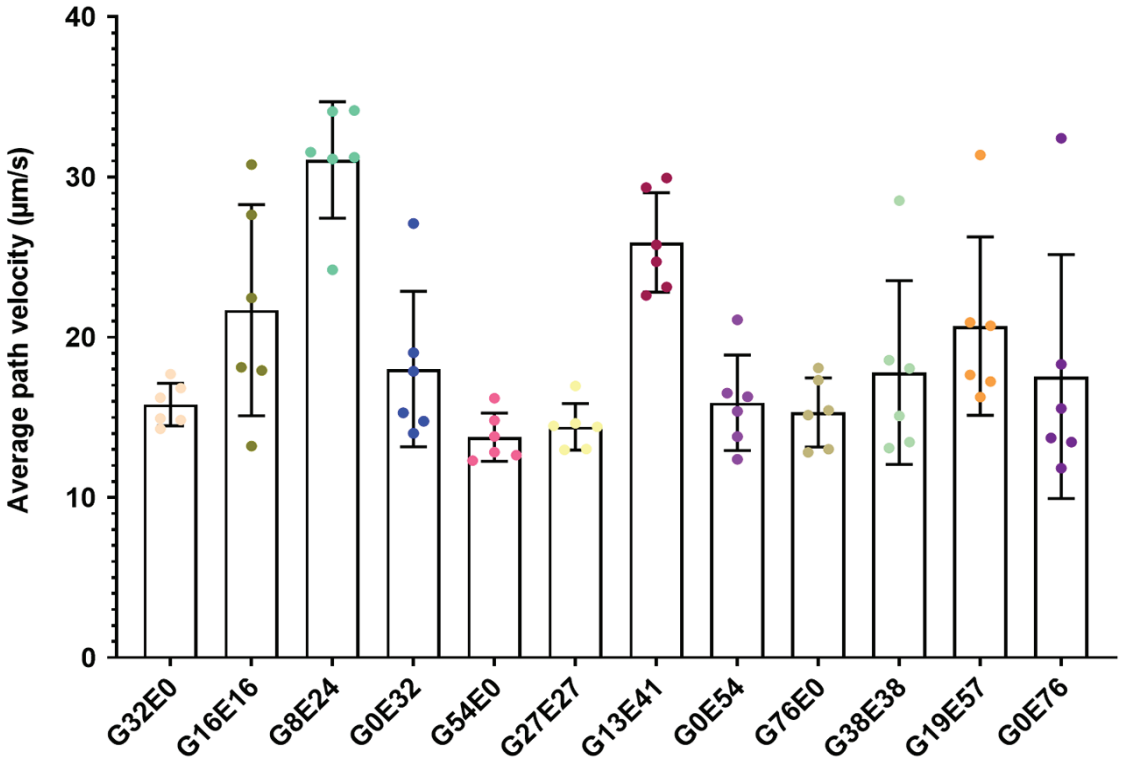

Figure 4
Effect of glycerol and erythritol on the average path velocity of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

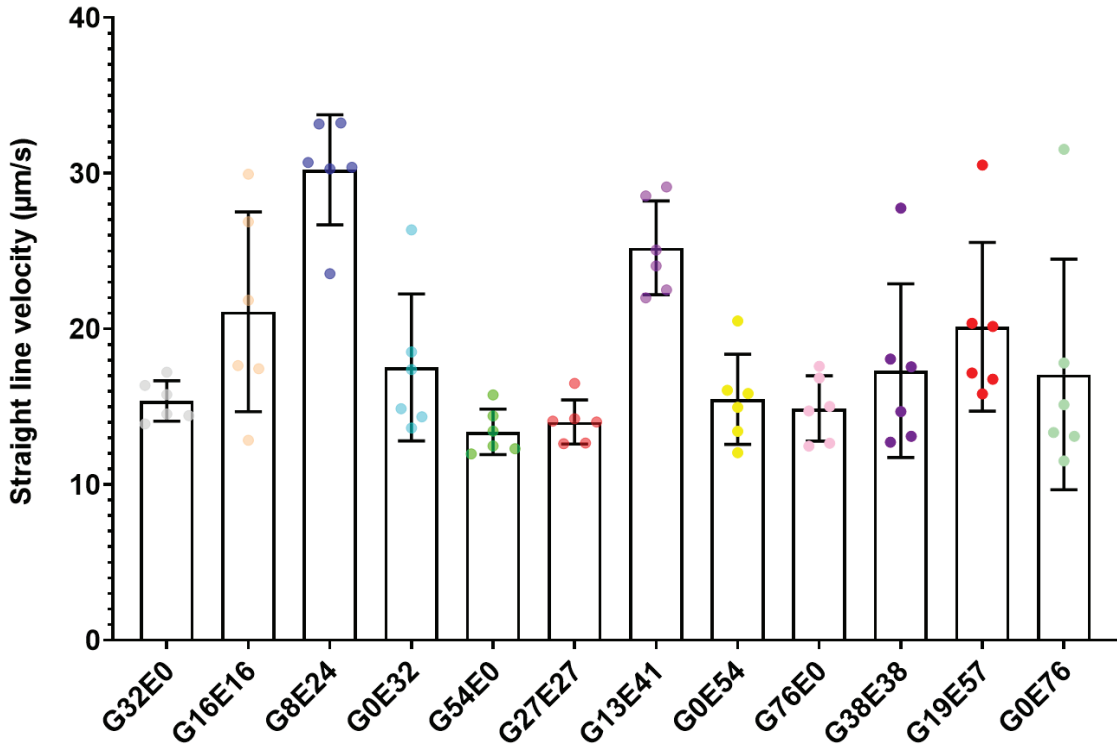

Figure 5
Effect of glycerol and erythritol on the straight line velocity of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

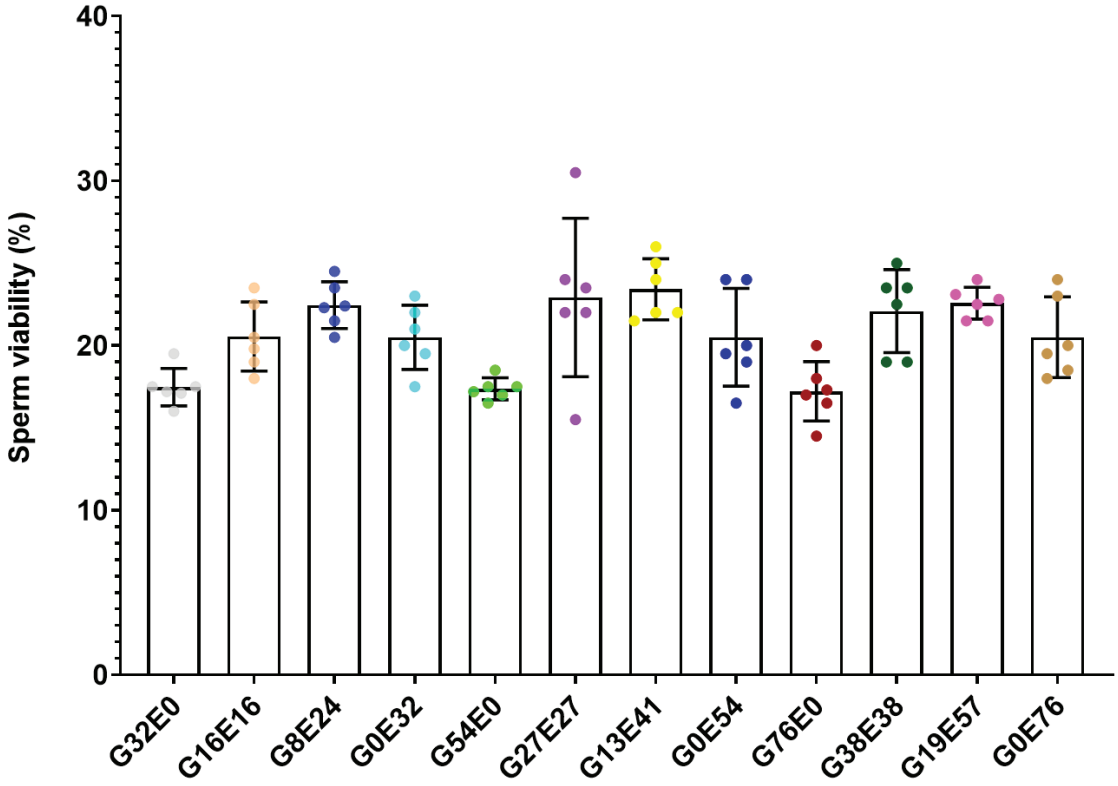

Figure 6
Effect of glycerol and erythritol on the viability of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

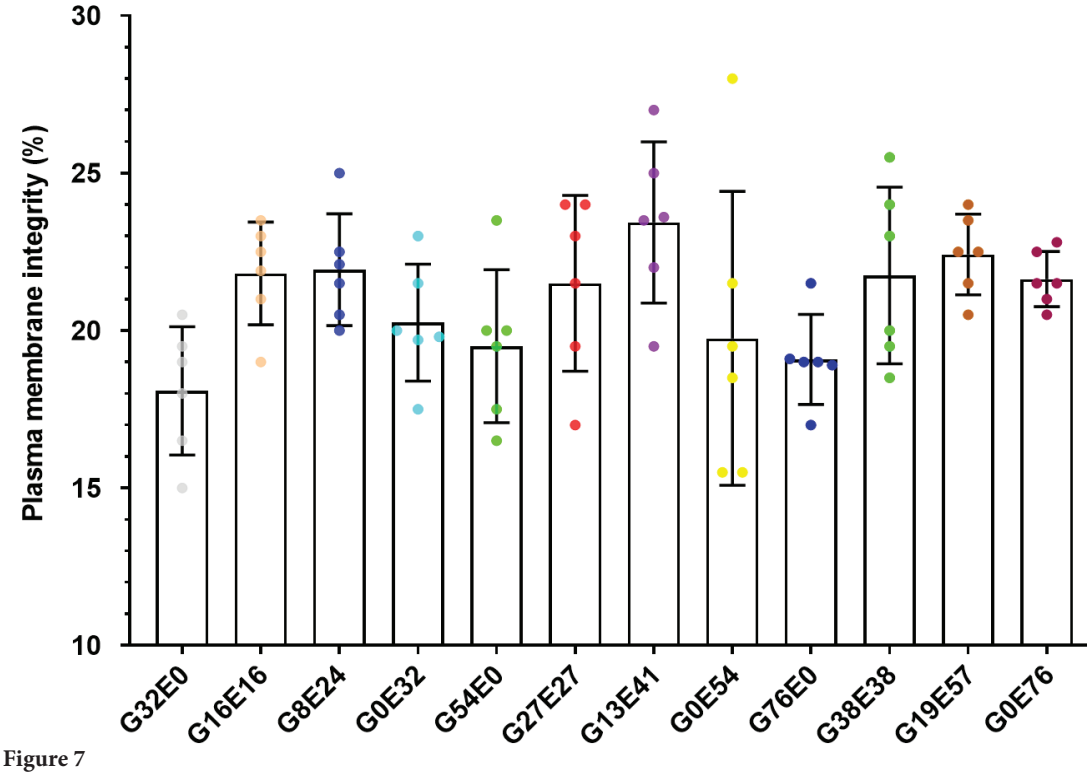

Figure 7
Effect of glycerol and erythritol on the plasma membrane integrity of ram spermatozoa 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

Table 1
Effect of glycerol and erythritol on acrosome integrity at 0, 3 and 6 h after thawing.

Treatments*	Acrosome integrity (%)		
	0(h)	3 (h)	6 (h)
G32E0	30.25^{abc} ± 0.96	25.50^{b} ± 0.89	20.8^{b} ± 0.77
G16E16	31.75^{abc} ± 0.72	27.58^{ab} ± 1.14	20.17^{b} ± 1.35
G8E24	29.50^{bc} ± 0.65	26.83^{ab} ± 1.41	24.57^{ab} ± 1.03
G0E32	29.92^{b}c ± 1.39	25.33^{b} ± 0.99	21.8^{3b} ± 1.35
G54E0	31.25^{abc} ± 1.70	27.50^{ab} ± 1.64	20.58^{b} ± 0.7^{0}
G27E27	35.83^{ab} ± 1.66	30.75^{ab} ± 2.11	24.42^{ab} ± 1.04
G13E41	36.83^{a} ± 2.18	31.92^{a} ± 1.69	27.41^{a} ± 1.87
G0E54	28.75^{c} ± 0.82	26.58^{ab} ± 1.00	23.50^{ab} ± 1.10
G76E0	29.25^{bc} ± 1.70	25.08^{b} ± 0.72	20.58^{b} ± 0.70
G38E38	32.67^{abc} ± 1.77	28.25^{ab} ± 1.70	24.58^{ab} ± 0.82
G19E57	27.83^{c} ± 1.46	24.75^{b} ± 0.63	22.33^{ab} ± 0.84
G0E76	29.92b^{c} ± 1.15	27.25^{ab} ± 0.67	21.33^{b} ± 1.33

* G3E0: 0.032 M glycerol, G3E1/2: 0.016 M glycerol + 0.016 M erythritol, G3E3/4: 0.008 M glycerol + 0.024 M erythritol, G3E1: 0.032 M erythritol, G5E0: 0.054 M glycerol, G5E1/2: 0.027 M glycerol + 0.027 M erythritol, G5E3/4: 0.013 M glycerol + 0.041 M erythritol, G5E1: 0.054 M erythritol, G7E0: 0.076 M glycerol, G7E1/2: 0.038 M glycerol + 0.038 M erythritol, G7E3/4: 0.019 M glycerol + 0.057 M erythritol, G7E1: 0.076 M erythritol.
$^{a-c}$ Different letters show significant differences ($p < 0.05$).

G13E41 than G32E0 at 6 h ($p < 0.05$).

Discussion

The methods of freezing ram spermatozoa have not well been optimized yet. The physical stresses of freezing process induce irreversible damage to the sperm organelles and enzymatic activities, which have been associated with sperm functionality and fertility [12]. This may be minimized by the inclusion of the suitable cryoprotectant for ram semen.

Results of this study showed that there was no difference between G32E0 and G0E32, G54E0 and G0E54 and also G76E0 and G0E76 on the total and progressive sperm motility, viability, membrane integrity and acrosome integrity. Consequently, in the process of freezing of ram spermatozoa, a complete replacement of glycerol with erythritol is possible. Similarly, erythritol is used for freezing bull and boar spermatozoa, human erythrocytes and some animal embryos [9, 13, 14, 15, 16, 17]. It has been mentioned that the cryoprotective effect of the cell-penetrating polyols are enhanced along with the increase in their hydroxyl groups [9]. When polyols are used, these substances replace the water around the phospholipid head groups and the hydrogen bond between the hydroxyl group of polyol and the phospholipid phosphate groups is formed that protect the membrane against the damage of freezing procedure [18]. Both erythritol and glycerol penetrate the membrane cell, but the permeability of membrane to glycerol is higher than erythritol, and glycerol has one less hydroxyl group than erythritol [9, 18]. Erythritol was as effective as glycerol for protection against freezing-induced damage to ram spermatozoa, although, their number of hydroxyl groups and ability to pass through the cell membrane were different.

Results showed that sperm viability was higher in G8E24, G13E41, G27E27 and G19E57 than G32E0 and G76E0. Membrane integrity was higher in G13E41 than G32E0. Moreover, progressive sperm motility was highest in G8E24 at 0.032 M cryoprotectant. At 0.032 and 0.054 M cryoprotectant, the highest of the total sperm motility was observed in G8E24 and G13E41, respectively. Consequently, the mixture of erythritol and glycerol appears to provide protection to ram spermatozoa against the physical stress of freezing. Based on our knowledge, erythri-

tol is free of side effects in regular use and also it is able to exert antioxidant activity in a cellular system [10, 11]. It has been indicated that erythritol was not mutagenic for bacterial cells and did not cause chromosomal damage to mammalian cells either *in vitro* or *in vivo* [19]. Moreover, it was illustrated that erythritol reduces the mitochondrial damage and increases the fertility of boar sperm after freezing-thawing [16]. On the other hand, glycerol is actively metabolised to formaldehyde, and high level of formaldehyde can destroy cell membranes [11]. Formaldehyde, highly reactive compound, is a strong inducer of apoptosis and lipid peroxidation [20]. Furthermore, it has been illustrated that removing glycerol via dialysis improve the fertility of frozen ram spermatozoa [5]. It was suggested that reducing the concentration of glycerol in the semen extender might be beneficial to the survival of spermatozoa when glycerol has been mixed with other cryoprotectants [21]. The combination of adonitol (up to 450 mM), as a low molecular weight polyol, and low levels of glycerol (1.5% v/v) improved the quality of pellet-frozen ram spermatozoa, whereas the high molecular weight polyols/glycerol combination had a detrimental effect [15]. Observations showed that the mixture of cryoprotective agents and saccharides provided better result than individual agents[22]. On the one hand, it was reported that if membrane integrity is supported by modifying the content of membrane cholesterol, the low concentration of glycerol (0.032 M) can protect ram spermatozoa during freezing [23]. Moreover, erythritol appears to stabilize membrane of bacteria [24]. According to the improvement of ram sperm quality caused by supplementation of semen extender with a low concentration of glycerol plus erythritol, therefore, the mixture of these polyols (at 0.032-0.054 M concentration) had a more protective effect on the frozen ram spermatozoa.

Our results showed that total sperm motility was higher in G8E24 than other treatments except for G13E41. Acrosome integrity was higher in G13E41 than G8E24 at 0 h, although, there was no difference between them at 3 and 6 h after thawing. At 6 h, acrosome integrity was higher in G13E41 than treatments containing only glycerol. Consequently, both the mixture of 0.013 M glycerol plus 0.041 M erythritol and 0.008 M glycerol plus 0.024 M erythritol improved longevity of frozen ram spermatozoa. Cryopreservation induces premature acrosome reactions [25]. Moreover, glycerol induces premature acrosome reaction and this lowered fertilizing capacity of the spermatozoa [5]. Furthermore, it was mentioned that glycerol had a detrimental effect on the acrosome integrity of ram spermatozoa [26]. Glycerol, as a formaldehydrogenic compound, may be considered as an exogenous source of formaldehyde [27]. The low concentration of formaldehyde increases intracellular calcium concentration in cultured hippocampal neurons via NMDARs and T-type Ca2+ channels [28]. Intracellular calcium is an important intracellular messenger and plays a key role in many physiological processes such as acrosome reaction [29]. Therefore, reducing the concentration of glycerol and replacing it with erythritol may improve the quality of the ram spermatozoa in the process of semen freezing.

In conclusion, our study demonstrates for the first time that ram spermatozoa are protected by erythritol against the physical stress of freezing. Moreover, the concentration of glycerol can be reduced by supplementation of semen extender with erythritol in semen cryopreservation. Furthermore, the quality of frozen-thawed ram spermatozoa can be improved by using the mixture of 0.013 M glycerol plus 0.041 M erythritol or 0.008 M glycerol plus 0.024 M erythritol.

Material and methods

Chemical reagents

The following chemicals and materials were used: meso-Erythritol (Sigma-Aldrich, St. Louis, MO, USA), Tris [hydroxymethyl] aminomethane, citric acid monohydrate, glucose, fructose and sodium citrate dihydrate, polyvinyl alcohol, glutaraldehyde, hoechst bisbenzimide 33258 (AppliChem GmbH, Darmstadt, Germany), potassium hydrogen phosphate, sodium hydrogen phosphate, sodium bicarbonate, ammonium sulfate, ammonium bicarbonate, sodium chloride, potassium chloride, methanol and glycerol 99.5% (Merck, Darmstadt, Germany), Alexa Fluor-488-PNA conjugate (Molecular Probes, Eugene, OR, USA).

Animals

This experiment was performed at University of Guilan, Faculty of Agricultural Sciences, Education Research and Practice Farm, South of Rasht (it is located at 37° 12′north latitude and 49° 39′east longitude). Four healthy mature Taleshi rams at age between 3 and 5 years were used. Rams were fed daily with a diet of 1300 g alfalfa hay, 620 g rice straw, 590 g barley and 100 g concentrates. Animals had free access to mineral supplement and fresh water. Animals cared for under experimental procedures and protocols approved by the Veterinary Organization of Iran.

Semen collection

Ejaculates (n=24) were collected from four rams by artificial vagina twice a week during the breeding season for six times. After ejaculation, the semen was diluted 1: 2 (v/v) with Tris diluents (300 mM tris [hydroxymethyl] aminomethane, 95 mM citric acid monohydrate, 28 mM D-glucose, 25 µg/mL gentamycin, pH 7.0) which containing 15% egg yolk. The samples were immersed in 33°C water and transferred to the laboratory by Styrofoam box within 10 min after collection [2].

Semen dilution, freezing and thawing

Upon reaching the laboratory, evaluation of samples was performed immediately. All diluted ejaculates were tested to possess an acceptable volume (> 0.5 mL), progressive motility (> 70%) and concentration (> 2.5 $\times 10^9$ sperm/mL).

In each session, the ejaculates were pooled, diluted up to 1.2×10^9 (cell/mL) by Tris-egg yolk (15%) and split into 12 equal parts. It was added to diluted semen 0.032 M, 0.054 M and 0.074 M glycerol and/or erythritol. The amount of 0.032 M glycerol (G32E0), 0.016 M glycerol and 0.016 M erythritol (G16E16), 0.008 M glycerol and 0.024 M erythritol (G8E24), 0.032 M erythritol (G0E32), 0.054 M glycerol (G54E0), 0.027 M glycerol and 0.027 M erythritol (G27E27), 0.013 M glycerol and 0.041 M erythritol (G13E41), 0.054 M erythritol (G0E54), 0.076 M glycerol (G76E0), 0.038 M glycerol and 0.038 M erythritol (G38E38), 0.019 M glycerol and 0.057 M erythritol (G19E57) and 0.076 M erythritol (G0E76) were added. Final concentration of sperm was 600×10^6 cells/mL. The diluted samples were packaged into 0.25 mL French straws, sealed with polyvinyl alcohol powder and cooled to 5□ over 2 h (0.25°C/min) and maintained at 5°C for 2 h. The straws were frozen in liquid nitrogen vapor, with the straws horizontally suspended 4 cm above the liquid nitrogen for 13 min, before being plunged into liquid nitrogen for storage [23]. Three straws for each replicate were thawed in a water bath at 37°C for 30s. Thawed semen incubated at 37°C for 6 h. Sperm motility, viability, functional membrane integrity and acrosome integrity were assessed immediately after thawing (0 h), subsequently after 3 h and 6 h post-thawing incubation.

Sperm assessment

The concentration of spermatozoa was determined by means of a Neubauer haemocytometer.

Total and progressive sperm motility were analyzed using a CASA system (Animal Version 6.51HFT CASA, Iran), digital colour camera (Samsung, Techwin, CO LTD, SCB-2000, Korea), phase-contrast microscope (GX Microscopes, Australia) equipped with heated stage set at 37°C. The sperm analyser was set-up as follows: frame rate – 50 Hz; minimum contrast – 50; low and high static-size gates – 0.53–4.45. Pre-warmed chambers slide (Sperm Processor, India) was loaded with 5 µL of a diluted sample at a concentration of 2×10^6 sperm/mL. Sperm motility parameters were recorded at 400× magnifications for 20 microscopic fields, at 37°C.

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane [2]. HOST was performed by incubating 5µL of semen with 50µL of a 100 mOsm hypo-osmotic solution (7.35 g sodium citrate dihydrate and 13.51 g fructose in 1000 mL distilled water) at 37°C for 30 min. One drop of the mixture was placed on a pre-warmed slide, covered with a cover slip and examined under a phase-contrast microscope (400×magnification). The sperm with swollen tails were considered intact. To assess the percentages of intact sperm, a total of 200 sperm were evaluated in at least five different microscopic fields.

Sperm viability was evaluated by fixed vital staining method [30]. Briefly, sample was mixed with an equal volume of a 2% glutaraldehyde solution (w/v) in phosphate-buffered saline (PBS), then it was mixed with an equal volume of 20 µg/mL bisbenzimide H33258. A smear was prepared after 10 min of incubation at room temperature. Two hundred spermatozoa per smear were evaluated in 3-7 different microscopic fields for each sample using an Olympus IX70, phase-contrast microscope (high-pressure mercury illuminator, UG1 excitation filter, U dichroic mirror, L420 barrier filter; Olympus, Tokyo, Japan). The procedure was performed by epifluorescence microscopy combined with bright-field illumination. Light intensity of the microscope was set at an optimum for visualization of both spermatozoa and fluorescence of H33258-labeled nuclei. Sperm showing partial or complete blue color was considered as dead, and sperm showing without color was considered to be alive.

Sperm acrosome integrity was estimated by staining with Alexa Fluor-488-PNA conjugate [2]. Briefly, sperm samples from each treatment were smeared on microscopic slides and air-dried. The samples were then fixed with methanol and kept at room temperature until staining procedure. For staining, ram sperm were incubated with 10 µg/mL Alexa Fluor-488-PNA in the darkness at 37°C for 30 min, washed with PBS and then analyzed under epifluorescence microscope (Olympus IX70) using an appropriate filter. In each sample, approximately 200 sperm were evaluated to determine the proportion of sperms with intact acrosome.

Statistical analyses

All data were analyzed by ANOVA followed by Tukey's test via the GLM procedure of SAS (version 9.1, SAS Institute Inc., 2002). The data of sperm motility, viability, function membrane integrity and acrosome integrity were analyzed by completely randomized design with 12 treatments in six replications. Differences were considered to be statistically significant at $p < 0.05$. Results were reported as means ± SE.

Author Contributions

M.A. performed the experiments. M.R.A.M. designed the research project and drafted the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

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RESEARCH ARTICLE

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A comparison study on the cow and mare milk-clotting activity of *Withania coagu-*

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ABSTRACT

The limitations of rennin application in cheese crafting usually urge the discovery of novel proteases. The *Withania coagulans* fruits are well-known for their caseinolytic activity in cheese production. The study aims to evaluate some of the factors affecting the milk-clotting activity (MCA) of *W. coagulans* fruit enzymatic extract in even and odd-toed hoofed-mammals milk. The extracts were prepared by distilled water and normal saline and their protein content were evaluated. The time necessary for the appearance of discernible and discrete particles in the mare and cow milk by the two concentrations of saline and hydro *W. coagulans* extracts (SE and HE, respectively) was assessed at 35 and 40°C while exposing different levels of calcium chloride. The interaction of these factors on MCA was evaluated using mixed-design ANOVA. Three significant interaction patterns considering the maximum number of factors were revealed ($p < 0.05$). The higher extract concentration and incubation temperature (40°C) was always effective in producing the utmost MCA in these interactions. The SE was faster than HE in milk clot formation. The cow milk was a more suitable substrate than mare milk for the enzyme activity.

Keywords

Withania coagulans, milk-clotting activity, cow milk, mare milk

Abbreviations

MCA: Milk-clotting activity
E1: 25% concentration of the enzymatic extract
E2: 100% concentration of the enzymatic extract
HE: Hydro extract of *Withania coagulans*
SE: Saline extract of *Withania coagulans*

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Introduction

Cheese production is one of the most important methods for preserving the main ingredients of milk [1]. The enzymatic gelatinization is a crucial stage in cheese manufacturing [2]. Mostly, specific proteases gelatinize and form the cheese clots by protein coagulation and the release of whey. Currently, animal, herbal, fungal, and microbial proteases are applied along with the recombinant enzymes in cheese manufacturing [3].

The most commonly known cheese rennet originates from an animal source and is being extracted from suckling calf abomasum. Despite the benefits of this kind of rennet and its growing demand, its production has decreased drastically due to the less young calves slaughtering, rising meat prices, the emergence of dangerous diseases such as mad cow disease and the increase of vegetarianism [4, 5].

Microbial rennets reduce cheese yield and produce bitter peptides due to the high and non-specific proteolytic activity [6]. Genetically engineered cheese rennets also have limitations for consumers, which are prohibited in Germany, the Netherlands, and France [7].

Considering the limitation of renin production and its rising prices, plant proteases can be a good alternative to renin. Traditionally, plant rennets have been used in cheese production for long times [7]. Recently, studies postulate that plant proteases can be an affordable substitute for other rennets. Despite extensive researches on the possibility of plant rennet application in the cheese industry, some results indicate that these rennets are sometimes unsuitable for cheese making due to high proteolytic activity and a bitter taste of the final product. Furthermore, non-specific casein hydrolysis and the reduction of curd strength in comparison with calf chymosin is another negative prospect of plant rennets [8, 9].

One of the plant proteases is originated from *W. coagulans*, and it seems that it does not have the undesirable properties of plant rennets [10, 11]. The zymographic analysis of this enzyme has shown that it is an aspartic protease with a molecular weight of about 35 Kilo-dalton [12]. *W. coagulans*, a plant belonging to the Solanaceae family, grows wild all over Pakistan as well as southwest of India and Afghanistan. In Iran, the distribution of this plant is limited to Sistan and Baluchestan Province [13, 14].

Unlike ruminants' milk, mare milk does not usually produce a firm clot after the addition of common rennets [15]. Therefore, introducing an enzyme with a proper mare milk clotting activity is important in the cheese industry. Various factors affect the rennet coagulation properties. Most of the coagulants act at 35-40°C to form semi-hard to hard cheese [16].

In addition to the type and concentration of a milk-clotting enzyme, calcium chloride ($CaCl_2$) has been shown to enhance the formation of casein clots [17, 18]. Elevation of Ca^{2+} and calcium phosphate colloids along with the hydrogen ion activity are the main reasons for this enhancement [19].

The object of this study was to evaluate the role of type (saline and hydro) and concentrations of *W. coagulans* enzymatic extracts in commencing the clot particles formation in even and odd-toed ungulates' milk (cow and mare) at different incubation temperatures and $CaCl_2$ levels.

Results

The total extracted protein from *W. coagulans* in saline was higher than the distilled water. The protein contents of the HE and SE assessed via UV spectrophotometer absorbance were 78.62% and 57.63%, respectively.

The total protein and casein content of the mare and cow milk were evaluated by a formol titration method and a Kjeldahl-linked assay (Figure 1). Holstein cow milk had an average total protein of 3.6%. In this study, the total protein of Holstein cow milk was 1.9% higher than Arabian mare milk. Furthermore, the total casein of cow milk was 2.9% which results in a 1.5% higher casein content than the mare milk.

In this study, the simple effects of 5 factors, i.e.: milk type (cow vs mare), the level of $CaCl_2$ in milk (0.5, 10, and 200mM), the type (HE vs SE) and the concentration (E1 and E2) of *W. coagulans* extract and the reaction temperature (35 and 40°C) were investigated on the start of milk clot formation (Figure 2). Considering the maximum number of factors, three

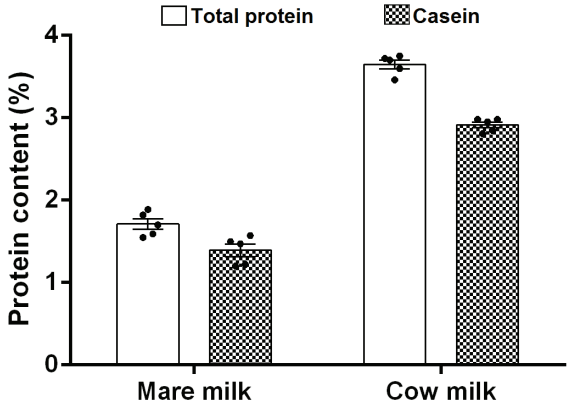

Figure 1.
The total and casein proteins concentration of the cow and mare milk (mean ± SEM; n = 5).

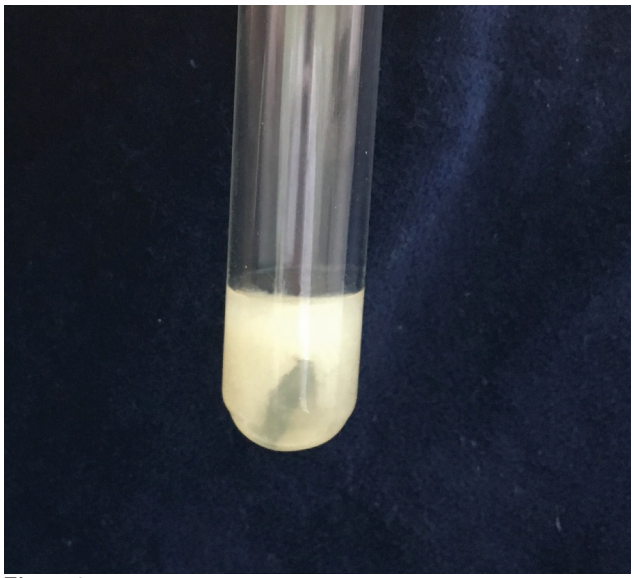

Figure 2.
The milk clot results from the *W. coagulans* extract.

significant interaction patterns (each containing four factors at the same time) were identified which affect the MCA.

In the first pattern, the four factors which comprise milk type, $CaCl_2$ level, *W. coagulans* extract concentration, and reaction temperature showed interaction with each other (p = 0.00025). Hence, the MCA was affected by the different levels of these four factors. The highest MCA level was 1824 units, which was observed due to the effect of 100% concentration of the extract on cow milk in the presence of 200 mM $CaCl_2$ at 40°C. The statistical differences of the interaction between the levels of these four factors are shown in Figure 3.

The second interacting pattern is between the four factors: milk type, reaction temperature, type and concentration of *W. coagulans* extract (p = 0.016). In this interaction, the highest MCA level (1324 units) comes from the effect of SE with 100% concentration on the cow milk at 40°C. The statistical differences between the levels of these four factors are shown in Figure 4.

Finally, the four factors of reaction temperature, $CaCl_2$ level, and type and concentration of *W. coagulans* extract demonstrated the third interaction pattern (p = 0.045). In this interaction pattern, the highest MCA level was 1310 units and was observed due to the effect of 100% of SE in the presence of 200 mM $CaCl_2$ at 40°C. The statistical differences between the levels of these four factors are shown in Figure 5.

Discussion

In the simultaneous assessment of all the three interaction patterns, it is found that the factors of temperature and the extract concentration play more important roles because these factors are being effective in all three patterns. Despite the effect of factors such as extract type, $CaCl_2$ level and milk type on MCA in some patterns, there is always an interaction pattern without the significant effect of these factors on MCA. For example, the extract type in the first interaction pattern, the level of $CaCl_2$ in the second interaction pattern and the milk type in the third interaction pattern (shown in the results) were not significant.

In this study, elevating the temperature from 35°C to 40°C increased the MCA. This increase in the activity may be due to the enzyme or substrate molecules. As the temperature rises, the rate of enzymatic reaction can also be increased due to the elevated kinetic energy of the substrate molecules. The number of substrate molecules with a kinetic energy of more than the energy barrier for product formation increases at higher temperatures. High substrate kinetic energy also increases their motion, and eventually the collision frequency of the molecules [20]. Beigomi and colleagues also showed that the activity of *W. coagulans* enzyme increases up to the temperature of 70°C [21]. Each protein has its own temperature susceptibility. The higher MCA of the enzyme at 40°C may also pertain to its steady 3D structure at this temperature. Generally, enzymes lose their 3D structure at high temperatures which results in their inactivity. For example, the optimal temperature for the activity of *Aspergillus niger* protease on shrimp peptone is 50°C [22].

Typically, MCA is dependent on the concentration of available enzymes [23]. The enzyme concentration can increase the amount of Kappa-casein proteolysis [24]. In this study, the higher extract (enzyme) concentration in cow milk always lowered the coagulation time and increased MCA (Figures 3; 4-B). The high concentration of *W. coagulans* extract increased MCA in mare milk at 40°C (Figure 3). Accordingly, the interaction of extract concentration and the reaction temperature sometimes plays an important role in MCA. Several models have been proposed for milk coagulation time versus enzyme concentration. In the first model, clotting time is inversely proportional to enzyme concentration [25], in the second model, the inverse clotting time is proportional with the concentration of enzyme [26], and in the third model, milk-clotting time is proportional to the inversed second root of enzyme concentration [27]. The higher concentration of *W. coagulans* extract reduces the time of skim milk clotting similar to other milk-clotting enzymes, including animal rennets (chymosin and pepsin), fungal rennet (*Mucor miehei* and *Mucor pusillus*) and the plant enzymes such as *Cyanara*

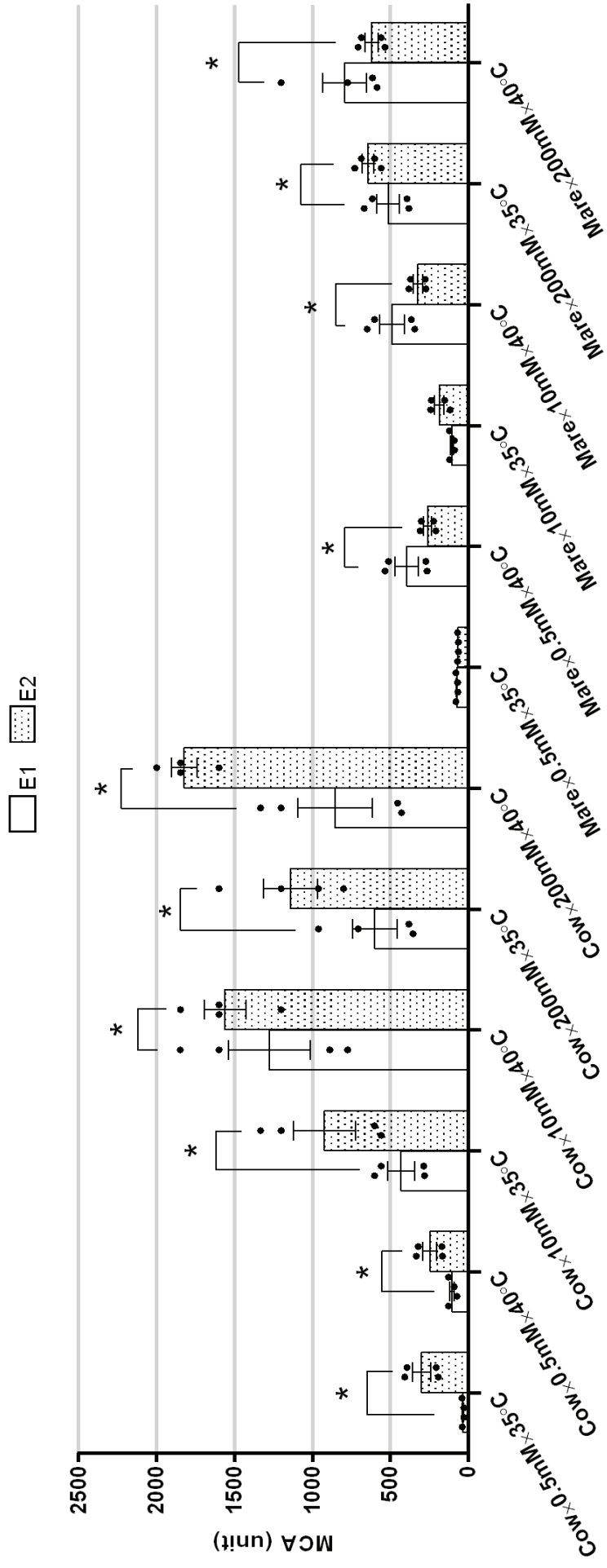

Figure 3
The MCA [mean±SEM; n=4] changes ($p < 0.05$) due to the concentrations of *W. coagulans* extracts (E1 and E2) in the presence of three $CaCl_2$ levels (0.5, 10, and 200 mM) in the mare and cow milk at 35 and 40°C.

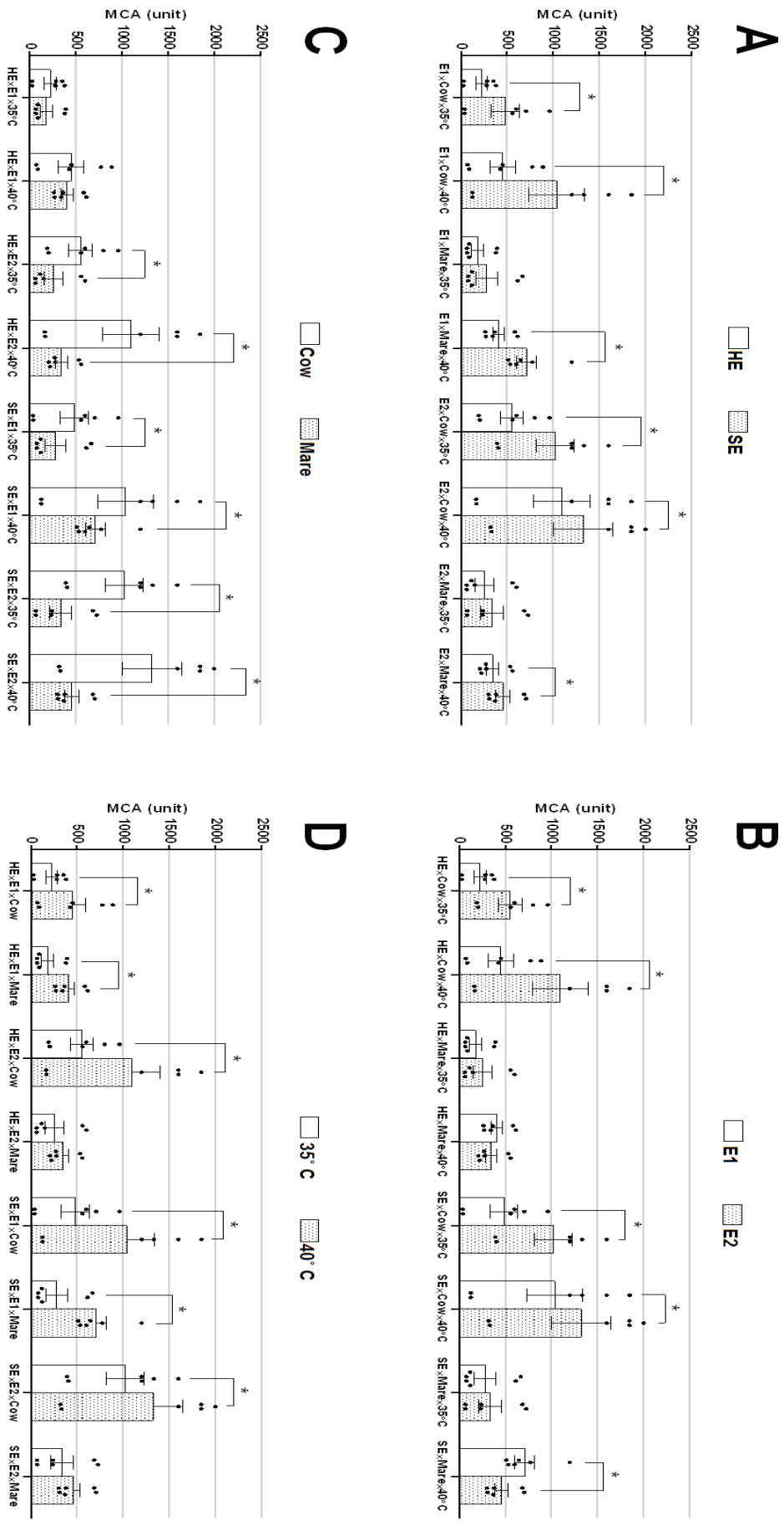

Figure 4
The interactions of (A) two types (HE and SE) and (B) concentrations (E1 and E2) of *W. coagulans* extracts on (C) mare and cow milk clot formation at (D) two temperatures (35 and 40°C) [mean±SEM; n=6] ($p < 0.05$).

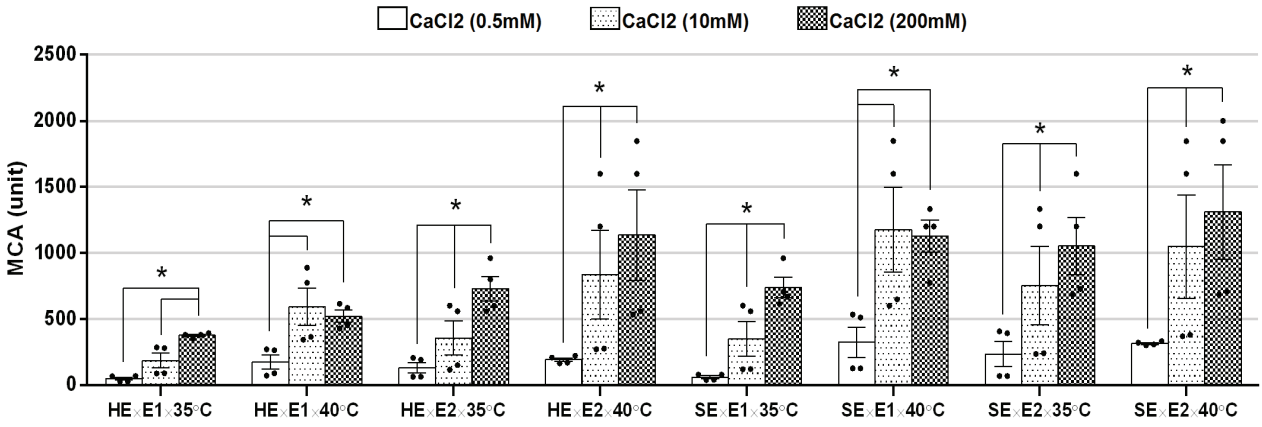

Figure 5.
The effect of three $CaCl_2$ levels (0.5, 10, and 200 mM) on the MCA [mean ± SEM; n=4] results of two types (HE and SE) and concentrations (E1 and E2) of *W. coagulans* extracts at 35 and 40°C ($p < 0.05$).

scolymus L. [23, 28, 29].

The SE always showed a higher clotting activity in the cow milk than the HE. While this extract in the mare milk exhibited more clotting activity than the HE only at 40°C (Figure 4-A). Some studies indicate that the protein's solubility increases at low salt levels [30]. For example, the solubility of the lysozyme protein increases in the physiological level of NaCl [31]. Chaotropic ions are known as 'salting-in' responsible ions due to their ability in increasing the solubility of proteins [32]. Divalent ions are more capable of protein salting-out than the monovalent ions (NaCl-forming ions) [33]. As shown, the higher protein content of SE than the HE may indicate a better enzyme extraction by normal saline. Traditionally and in some studies, saline is used to extract the enzyme of this plant for cheese production [34-36].

The milk type is effective on MCA in the first and second interaction patterns. Contrary to these two patterns, milk type does not play a role in the third interaction pattern (Figure 5). On many occasions, e.g.: SE and the high concentration of HE, the MCA in cow milk was higher than the mare milk (Figure 4). However, there was not any significant difference between the mare and cow milk clot formation by the 25% concentration of HE (Figure 4-C). The *W. coagulans* enzyme shows caseinolytic activity [12]. The amount of cow milk casein is higher than mare milk. The total mare milk proteins contain 40-60% casein. This proportion does not change extensively during the lactation period. Other dairy species such as cattle have higher casein levels in their milk [37]. The ratio of kappa-casein levels in mare milk is lower than ruminants [38]. Robitaille et al. showed that increasing the kappa-casein to total milk casein ratio accelerates cheese clot formation and enhances the clot consistency [39]. Furthermore, the temperature sensitivity of mare milk is lower than cow milk [40]. So that the cow milk at a higher temperature is more prone to clotting by the enzyme. Notably, in the evaluation of MCA in this study, milk type on some occasions (i.e.: the third interaction pattern) does not show any interaction with the temperature.

In this study, mostly the MCA enhanced as the level of $CaCl_2$ increased (Figure 5). $CaCl_2$ induces casein clot formation in milk through various mechanisms. It reduces the time for curd formation by changing the hydration forces between casein micelles and water [41]. Furthermore, increased H^+ and Ca^{2+} levels and their association with casein phosphate and carboxylic groups are needed to initiate clot formation [24]. The addition of $CaCl_2$ supplements reduces the time of clot formation by calf rennet, rennet-pepsin, *Mucor miehei* rennet and the *Bacillus sphaericus* clotting enzyme [42, 43]. Addition of 20–200 mM $CaCl_2$ in milk causes a 70°C heat-induced coagulation [44]. Here, there was no significant difference between the presence of 10 and 200 mM of $CaCl_2$ in MCA with a low concentration of the extracts at 40°C, whilst in other situations, the higher $CaCl_2$ level increased MCA (Figure 5). High levels of $CaCl_2$ might also increase casein clots production due to decreased sample pH [16]. Here, mostly the MCA was higher with 10 mM $CaCl_2$ rather than 0.5 mM $CaCl_2$. Adding 10mM $CaCl_2$ into milk enhances curd firmness about 81%. This is especially important in preventing weak casein clots [17].

In summary, it has been shown that saline (0.85%) extract of *W. coagulans* fruit has a higher protein content and higher MCA than the HE. The evaluations revealed that raising the temperature from 35°C to 40°C and increasing the extract concentration significantly elevates MCA. These factors have interactions

with other factors such as the type of milk, the type of extract and the $CaCl_2$ level on affecting the MCA level. The *W. coagulans* extract is likely to have more clotting activity on cow milk than mare milk due to its higher casein content and the casein's structure. Although the fruit extract is capable of cow milk clotting, the parameters such as the cheese yield and cheese sensory properties should be evaluated after using SE on different ruminants' milk at 40°C through cheese crafting studies.

Material and methods

Plant and milk preparation

The fruits of *W. coagulans* were prepared from Sistan and Baluchestan Province, Iran. The fruits were kept in a dark zip-sealed plastic bag in a cool place until the usage. Fresh cow milk was obtained from the Holstein breed and the fresh mare milk was from the Arab horse during the warm season. The two types of milk were pasteurized at 73°C for 15 seconds and cooled to 4°C. The milk was used in the assay within 2 days after pasteurization.

Preparation of W. coagulans extracts

The skin and pulp of *W. coagulans* fruits were carefully separated from the seeds. These compartments were mixed and powdered by mortar and pestle. The HE and SE of *W. coagulans* were extracted by distilled water and normal saline (0.85%). The fruit powder and the solvent (1:6) were mixed thoroughly in a shaking incubator (JSR, JSSI-100C compact shaking incubator) at 4°C for 24h. Homogenates were filtered (Whatman No. 1) and the filtrates were assayed immediately as the crude extracts [34].

Protein assessment of the W. coagulans extracts

The protein concentration of the extracts was evaluated by UV spectrophotometer absorbance [45]. Various dilutions of the HE and SE were prepared and their absorbance was measured at 260 and 280 nm spectrophotometrically (Shimatzo, Japan, UV-1201). The protein concentration of the extracts was calculated by Equation (1) as follows:

Equation (1): Protein concentration (mg/mL^{-1}) = (Abs.280 × 1.55) – (Abs.260 × 0.76)

Total protein and casein contents of cow and mare milk

Cow and mare milk total protein was evaluated via a titration method proposed by Pyne [46]. To assess the casein content of milk, 50 mL of warm water (40°C) and 0.5 mL of acetic acid solution (10%) were added chronologically to 5 g of milk. After 10 minutes, the milk blend was mixed with 0.5 mL of sodium acetate (1N) and allowed to cool at room temperature. The mixture was filtered through filter paper (Whatman No. 1) while its beaker was washed three times with distilled water. The filter paper containing casein precipitates was digested in a Kjeldahl digestion flask and its total nitrogen was further assessed via the Kjeldahl method. Casein content of the samples was calculated using 6.38 as the protein factor [47].

Preparation of cow and mare skim milk powder

The fresh milk was centrifuged at 4°C (5000 rpm / 15 min.) in falcon tubes (Universal 320R centrifuge, Hettich, Tuttlingen, Germany) and further defatted manually. This procedure was repeated twice and the skim milk was allowed to shadow-dry on a plate for 24h. The dried skim milk was powdered by mortar and pestle and kept at 4°C until usage within 7 days.

Evaluation of the extracts' MCA

The milk clot formation of HE and SE extrcats in cow and mare were measured at 35 and 40°C. The Arima et al, 1970 and Beigomi et al, 2014 methods were used to measure the MCA of the extracts with some modifications [21, 48]. Three different levels (0.5, 10 and 200 mM) of $CaCl_2$ (Merck, 64271) were mixed thoroughly with 10% reconstituted skim milk powders. Afterward, the reconstituted skim milk containing $CaCl_2$ was warmed to 35 or 40°C for 5 min in waterbath (Memmert WNB-29, Germany). Two concentrations (E1 and E2) of the crude HE and SE were prepared using their own solvents. The extract and the warmed reconstituted skim milk (1:10) were mixed gently. The time of the first visible milk clots formation was recorded for each experiment. Each experiment was performed at least in triplicates. The milk without the extract was considered as the negative control while distilled water and saline were also considered as the solvent control in which produced no clot at the aforementioned temperatures. The MCA of the extracts was calculated using the following formula (Equation 2):

Equation (2): MCA (units) = 2400/T × S/E

Where "T" is the time of clot formation (seconds), "S" represents substrate volume (mL) and "E" stands for the *Withania* enzymatic extract volume (mL).

Statistical analysis

After performing descriptive statistics, the interaction of different factors (milk types, extract types, extract concentrations, $CaCl_2$ levels, and incubation temperatures) on the MCA was evaluated using a mixed-design ANOVA with a significant level of 0.05. The Syntax results are presented with Bonferroni correction.

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Author Contributions

H.E. conceived and designed the experiments. H.E., F.H. and S.M. performed the experiments. H.E. and F.H. analyzed the data and wrote the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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High prevalence of *Prototheca spp.* and isolation of fungal species in milk samples from cows suffering from mastitis in Mashhad city, northeast Iran

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ABSTRACT

The aim of this study was to investigate the fungi and algae isolated from milk samples in dairy cows with clinical and subclinical mastitis from dairy farms around Mashhad, Iran. A total of 503 milk samples were obtained from 10 industrial dairy farms. All samples were simultaneously cultured on the surface of Blood agar, Macconkey agar, and Sabouraud dextrose agar supplemented with chloramphenicol. Fungi and algae were identified using phenotypic characteristics. In the examined samples, the bacterial contamination (338 out of 503 samples; 67.20%) was the most dominant followed by algae (93 out of 503 samples; 18.5%), filamentous fungi (32 out of 503 samples; 6.4%) and yeast fungi (26 out of 503 samples; 5.2%). *Penicillium spp.* (2.8%), *Aspergillus spp.* (2.6%), *Cladosporium spp.* (1.2%), *Geotrichum spp.* (0.4%), *Ulucaladium spp.*, *Scopolariopsis spp.* and *Alternaria spp.* (0.2% each), *Cryptococcus neofermenes* (3%), *Candida spp.* (1%), *Trichosporon spp.* (0.8%) and *Rhodoturula spp.* (0.4%) were the filamentous and yeast fungi isolated from milk samples. Of the 93 algae isolated from 503 milk samples (18.5%), 83 (16.5%) samples were positive for *Prototheca zopfii* and 10 (2%) samples for *Prototheca wickerhamii*. According to the results of this study, yeast fungi, filamentous fungi and algae, especially *Prototheca spp.* are important contaminant factors in milk and contribute to clinical and subclinical bovine mastitis. Therefore, proper sanitation practices and management of dairy herds and judicious use of antibiotics is essential to control the fungal and algal contamination both in the environment and the breast.

Keywords

Mastitis, Cattle, Fungi, Algae, Prototheca spp.

Abbreviations

spp.: Species
SCC: Somatic cell count
CMT: California Mastitis Test

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Introduction

Bovine mastitis has been defined as an inflammation of the mammary gland usually as a consequence of microbial infection. Mastitis in cattle is a serious problem which causes considerable economic losses in dairy cattle herds. The most common etiological factors are bacteria followed by mycoplasmas, viruses, fungi and algae [1].

Mastitis is usually transmitted through contaminated milking machines and milker's hands or other materials. Treatment is possible with long-acting antibiotics, but milk from such cows is not marketable until drug residues have left the cow's system. Antibiotics may be administered systemically, or they may be applied locally by upward force through the teat canal. Antibiotic therapy, without identifying the mastitis-causing organisms, is frequently the veterinarian and dairy farmer's first choice of treatment for diseased cows. As a result, cases of mastitis (including fungal mastitis) that are refractory to any type of treatment occur frequently [2-4].

Most common isolated fungi in cases of mycotic mastitis are the yeasts from genera Candida and Cryptococcus, precisely two species Candida albicans (C. albicans) and Cryptococcus neoformans (Cry. neoformans). Candida is a commensal of mucocutaneous areas, particularly of the intestinal and genital tracts. Yeast mastitis can emerge like clinical, subclinical, chronic and sometimes acute mastitis. The ways of transmission is almost always connected with administration of medicaments in udder or with surgical or other procedure on tit and so on. There are no differences between clinical manifestation of bacterial mastitis and yeast mastitis. Only by microbiological examination one can determine yeast mastitis. Prototheca is a genus of algae in the family Chlorellaceae. All species within this genus, even though classified as green algae, have forfeited their photosynthetic ability and have switched to parasitism [1, 5, 6].

Prototheca species (spp.) are colorless algae that can cause mastitis in dairy cattle. They are widespread in housing areas, pens and pastures used by dairy cattle. Most infections are clinical and remain as chronic infections. Prototheca spp. are often associated with wet areas containing decaying manure and plant matter [7, 8]. Prototheca infections are thought to occur when the teats of cows are exposed to high populations of algae in environmental sites during the milking intervals. Spread during milking time is not significant. However, new Prototheca infections can occur in situations where a high percentage of cows are infected with Prototheca and milking techniques are poor. Most mammary infections with Prototheca are clinical with the milk being grossly abnormal but without severe systemic signs such as off feed, depression or a high fever. Non-clinical outbreaks have been marked by normal milk with many quarters or cows with somatic cell count (SCC) greater than 1,000,000. In addition, most cows with protothecal infections will have reduced milk production [8].

Studies on fungal and algal infections of the mammary gland in cows are increasingly common due to their growing incidence. Therefore, in the present study we evaluated the isolation and frequency of fungal and algal species in dairy cows with clinical and subclinical mastitis in Mashhad, northeast of Iran.

Results

Out of 503 samples (232 from clinical cases and 271 from subclinical cases), 381 samples (75.74%) were positive for fungal, algal and bacterial contamination. In the examined samples, the bacterial contamination (338 out of 503 samples; 67.20%) was the most dominant followed by algae (93 out of 503 samples; 18.5%), filamentous fungi (32 out of 503 samples; 6.4%) and yeast fungi (26 out of 503 samples; 5.2%). There was no significant difference in bacterial/fungal/algal contamination between the clinical and subclinical mastitis cows ($p > 0.05$).

Overall, *Penicillium* spp. (2.8%), *Aspergillus* spp. (2.6%), *Cladosporium* spp. (1.2%), *Geotrichum* spp. (0.4%), *Ulucaladium* spp., *Scopolariopsis* spp. and *Alternaria* spp. (0.2% each), *Cryptococcus neoformans* (3%), *Candida* spp. (1%), *Trichosporon* spp. (0.8%) and *Rhodoturula* spp. (0.4%) were the filamentous and yeast fungi isolated from milk samples.

The culture-positive results obtained for *Candida* spp. showed that *C. albicans*, *Candida krusei* (*C. krusei*) and *Candida glabrata* (*C. glabrata*) were isolated from 1 (3.8%), 1 (3.8%) and 3 (11.5%) out of 26 samples, respectively.

Algal contamination was observed in only 3 dairy farms (30%). In dairy farm No. 1, *Prototheca zopfii* (*Pro. zopfii*) and *Prototheca wickerhamii* (*Pro. wickerhamii*) were isolated form 14 (15.21%) and 2 (2.17%) samples, respectively; whereas in dairy farm No. 2, *Pro. zopfii* and *Pro. wickerhamii* were isolated from 17 (20.73%) and 1 (1.21%) samples and in dairy farm No. 4, they were isolated from 52 (25.49%) and 7 (3.43%) samples, respectively (Table 1).

Figures 1 and 2 and Table 1 show the frequency of fungal and algal isolates in bovine mastitis of dairy farms in suburb of Mashhad, Iran. Two dairy farms had no fungal and algal contamination. Dairy farm number 4 had the most frequency for yeast and algal contamination and dairy farm number 8 had the most frequency for mold contamination.

From 381 culture-positive milk samples, mixed

and single cultures were found in 98 samples (24.72%) and 283 samples (74.27%), respectively. Table 2 show the microorganisms isolated from mixed cultures in examined samples.

Discussion

Conditions decreasing the resistance and susceptibility of cow udders to inflammations can be e.g. prolonged intra-udder antibiotics administration, increased incidence of udder mycosis results from mineral-vitamin deficiencies, antioxidant deficiencies, imbalanced diet, poor environmental conditions and even weather changes [10].Our observations concerning higher incidence of mycotic mastitis in cows treated with antibiotics confirm the earlier results of other authors [11-13].

In general, fungi are normal flora of the soil and

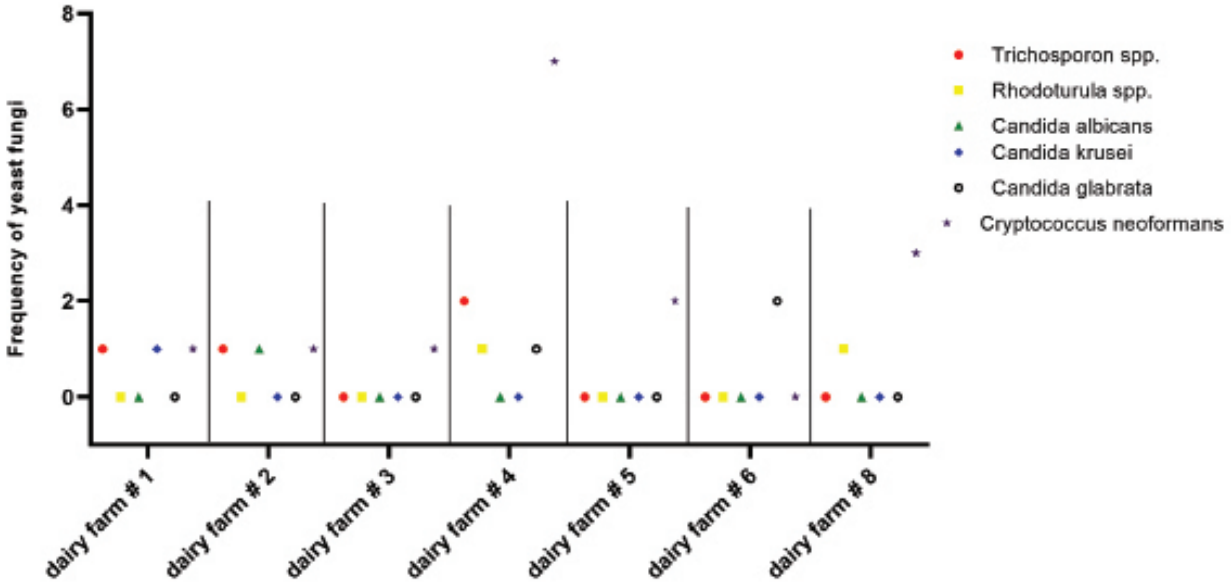

Figure 1.
The frequency of yeast fungi isolated from dairy cows with clinical and subclinical mastitis in different dairy farms in suburb of Mashhad, Iran.
* Three dairy farms had no yeast fungi contamination.

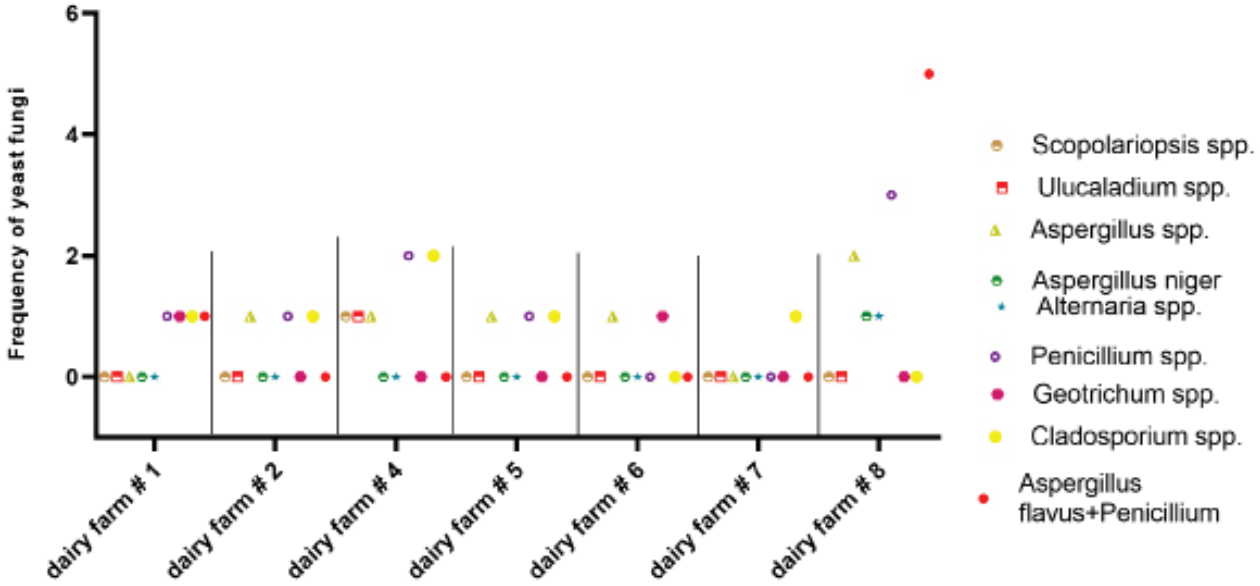

Figure 2.
The frequency of mold fungi isolated from dairy cows with clinical and subclinical mastitis in different dairy farms in suburb of Mashhad, Iran.
* Three dairy farms had no mold fungi contamination.

Table 1
The frequency of algae isolated in examined samples in different dairy farms in suburb of Mashhad, Iran.

Dairy farm	Number of samples	*Pro. zopfii*	*Pro. wickerhamii*	Total
		Number (%)	Number (%)	Number (%)
1	92	14(15.21)	2(2.17)	16(17.39)
2	82	17(20.73)	1(1.21)	18(21.95)
3	22	0 (0)	0 (0)	0 (0)
4	204	52(25.49)	7(3.43)	59(28.92)
5	22	0 (0)	0 (0)	0 (0)
6	20	0 (0)	0 (0)	0 (0)
7	6	0 (0)	0 (0)	0 (0)
8	44	0 (0)	0 (0)	0 (0)
9	2	0 (0)	0 (0)	0 (0)
10	9	0 (0)	0 (0)	0 (0)
Total	503	83(16.50)	10(1.98)	93(18.48)

Table 2
The frequency of the microorganisms isolated from mixed cultures in examined samples

Microorganisms in mixed cultures	Total
Penicillium spp. + bacteria	4 (0.79)
Geotrichum spp. + bacteria	3 (0.59)
Cladosporium spp. + bacteria	2 (0.39)
Aspergillus flavus + *penicillium spp.* + bacteria	4 (0.79)
Cladosporium spp. + bacteria + *Pro. zopfii*	5 (0.99)
Aspergillus niger+ *Cry. neoformans* + bacteria	1 (0.19)
Trichosporon spp. + bacteria	1 (0.19)
Rhodotorula spp. + bacteria	3 (0.59)
C. glabrata +bacteria	1 (0.19)
Cry. neoformans+ bacteria	2 (0.39)
Cry. neoformans + bacteria+ *Pro. zopfii*	8 (1.59)
Cry. neoformans + *penicillium spp.* + bacteria	1 (0.19)
Rhodotorula spp. + bacteria + *Pro. zopfii*	1 (0.19)
Pro. zopfii + *Cry. neoformans*	1 (0.19)
Pro. zopfii +bacteria + *C. albicans*	1 (0.19)
	2 (0.39)
Pro. zopfii + *C. glabrata* + bacteria	1 (0.19)
Pro. zopfii+ bacteria	51 (10.13)
Pro. wickerhamii + bacteria	6 (1.19)
Total	98 (19.48)

rarely cause mastitis but sometimes can occur in epizootic proportions [14], especially in farms with poor environmental and hygienic conditions as well as the reduction in animal's defense mechanisms [14, 15]. Poor quality of materials used as bedding (e.g. straw) with high humidity can be the source of fungi causing mastitis in cattle [14]. Fungi are also the reason of udder inflammation when udder is washed with water but is not dried [16].

Earlier studies conducted from 1982 to 1992 showed that about 6% of mastitis cases were caused by yeasts [17]; so that an important increase in the number of udder infections caused by *Candida* spp. and other yeasts was reported in various countries in recent years [15, 18-20].

The current study showed 11.53% fungal contamination (including 6.4% mold and 5.2% yeast) in milk samples from cattle with clinical and subclinical mastitis in the examined dairy farms.

In different studies, fungal contamination in cattle mastitis is variable. In this regard, in Tehran, Iran a comprehensive study was reported on the isolation of fungi by Talebkhan Garoussi et al. on milk of healthy, clinical and subclinical mastitis of dairy cows. They isolated different fungi from cows with clinical (14%), subclinical (18%) mastitis and healthy animals (15%)

[21]. Rasouli in Tabriz, Iran showed that 12.07% of milk samples of cows with mastitis had fungal contamination [22]. Batavani et al in Urmia, Iran isolated fungi (7.5%) (including 4.16% mold and 3.33% yeast) from milk of dairy cows with clinical and subclinical mastitis [23]. Several studies have been conducted on the isolation of fungi in other countries with rates of 17.3% in Brazil, 24.24% in Poland, 64% in India, and 6% in Serbia [15, 24-26].

Our results are considered to be the first report on the high prevalence of *Prototheca* spp. (18.5%) (including 16.5% P*rototheca zopfii* and 2% *Prototheca wickerhamii*) in examined milk samples in Mashhad, Iran. Poor environmental conditions, inappropriate milking hygiene and prolonged antibiotic therapy can be the reason of increased protothecal mastitis occurrence that can reach even over 30% [27]. Studies carried out by Krukowski et al. (2006), Lassa et al. (2013), Milanov et al. (2014) and Jagielski et al. (2019) revealed that *Prototheca* spp. was responsible for 0.35%, 0.9%, 4.6% and 11.3% of mastitis cases, respectively [15, 18, 20, 28]. Presence of the algal species *Prototheca zopfii* was demonstrated in analyzed samples from cows with mastitis in different papers [18, 27, 29-31]. In the present study, the investigation in examined dairy farms showed that feeding milk cattle containing fungi and algae (e.g. sugarcane bagasse) have increased the occurrence of protothecal mastitis.

According to the results of this study, yeasts and molds and algae, especially *Prototheca*, are important milk contaminants and contribute to the development of clinical and subclinical mastitis in dairy cattle. Compliance with the hygiene and management principles of dairy herds is essential for the control of fungal and algal agents in the environment as well as in the breast in order to prevent the development of clinical and subclinical fungal and algal mastitis and subsequently to prevent its economic losses and protect the community health.

Material and methods

During spring and summer of 2018, a total of 503 milk samples were collected from cows with clinical and subclinical mastitis from 10 industrial dairy cattle herds in suburb of Mashhad, Iran. Cows with clinical and subclinical mastitis were determined by clinical symptoms and using a California Mastitis Test (CMT), respectively [8, 9].Cows were not taken antibiotic(s) before sampling. Before sampling, the teats were washed, cleaned and disinfected using 95% alcohol. The first two stripping of milk were discarded. 15ml of milk was collected from each animal in sterile tube and transported to the laboratory under ice, and kept at 4°C until processing (no longer than 24 h after collection). All samples were simultaneously cultured on the surface of Blood agar (Merck Co., Darmstadt, Germany), Macconkey agar (Merck Co., Darmstadt, Germany) and Sabouraud dextrose agar (Merck Co., Darmstadt, Germany) supplemented with chloramphenicol. Plates were then incubated aerobically at 37°C for 24-48 h for bacteria growth and 25°C for 2 weeks for fungi and algae. Bacterial media were only studied based on the presence or absence of bacterial growth. Filamentous fungi were identified by standard mycological techniques based upon gross cultural and microscopic morphology. Yeast and algal colonies were identified on the basis of macro- and micromorphological characteristics, and on the basis of physiological characteristics, such as melanin production on Caffeic Acid Ferric Citrate test agar (HiMedia, India), presence of capsule by Nigrosin (Merck Co., Darmstadt, German) stain, urease production on urea agar medium (Merck Co., Darmstadt, Germany), the germ tube test, micromorphological analysis on corn meal Tween 80 agar, growth in CHROMagar candida (CHROMagar Co., Paris, France) and API 20 C AUX system (BioMérieux, France). The data were analyzed using the Chi-square and Fisher Exact tests. A *p*- value less than 0.05 was considered significant.

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Author Contributions

M.L. performed the experiments and drafted the manuscript. S.E. designed and conducted the study, analyzed the data, drafted and reviewed the manuscript. B.K. supervised sample collection.

Conflict of Interest

The authors declare no conflict of interest.

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Case-control study on risk factors associated with brucellosis in aborted cattle of Jimma zone, Ethiopia

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ABSTRACT

Brucellosis is one of the most important causes of abortion in cattle resulting in significant economic losses and public health concerns in the developing countries. A case-control study was conducted from October 2016 to October 2017 to investigate risk factors of brucellosis in aborted cattle in Jimma zone. During the study period, 141 cases and 282 controls were selected to assess and compare the presence of anti-Brucella antibodies between cases and controls. Cattle that had experienced abortion were defined as cases, whereas controls were cattle that had no record of abortion. Sera samples were collected from both cases and control cattle groups for laboratory tests (serological test). The existence of the anti-Brucella antibodes in serum samples was first tested by the Rose Bengal Plate test, and the all positive samples were confirmed using the complement fixation test. An overall of 4.02% seroprevalence of brucellosis was recorded in the study areas. Antibody against *Brucella* organism was higher among cases (6.38%) than controls (2.84%). Multivariable logistic regression analysis identified age (OR 14.16, CI= 2.91-28.84), breed (OR 5.36, CI= 1.76-11.33), herd size (OR 11.82, CI= 1.31-16.17) and species composition (OR 5.10, CI=1.49-13.43) as risk factors ($p < 0.05$) for *Brucella* seropositivity. This study documented the occurrence of cattle brucellosis in study areas. Thus, applicable control methods and creating public awareness on the zoonotic transmission of brucellosis should be conducted. Moreover, further study considering more causes should be carried out to identify the specific causes of abortion in cattle for the preparation of the appropriate vaccine.

Keywords

Risk factors, Brucellosis, Cattle, Ethiopia

Abbreviations

CFT: Complement fixation test
CI: Confident interval
D: Design effect
OR: Odd ratio
ROC: Receiver operating characteristic
NVI: National veterinary institute
RBPT: Rose Bengal plate test

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Introduction

Abortion in cattle is defined as loss of pregnancy within the day 42 to day 260 of gestation [1]. Termination of pregnancies before day 42 is typically referred to as early embryonic death, whereas a calf that is born dead between day 260 and the full term is mentioned as stillbirth [2]. Abortion results from infectious agents (bacteria, viruses, protozoa, and fungi) and non-infection causes such as heat stress, nutritional deficiencies, trauma, toxic substances, etc [3,4]. The infectious agents result in extensive economic losses, showing the requisite for control measures to prevent infection cause of abortion [5].

Among infectious causes, brucellosis is one of the most important causes of abortion in cattle and challenges to dairy industry [6]. It results in huge economic losses and public health concerns worldwide [7, 8]. Brucellosis in cattle is primarily caused by *Brucella abortus*, and *B. melitensis* and *B. suis* are seldom cause the disease in cattle. This disease is characterized in cattle by causing abortion, retained fetal membranes and infertility [9]. Contact with aborted cow, aborted fetus, or the contaminated fomites are the major routes of *Brucella* transmission [10]. Risk factors such as increased herd sizes, increased age, sex of cattle and husbandry practices are identified as precipitating factors for the occurrence of diseases in cattle [11, 12, 13, 14].

Brucellosis has higher incidence in a mixed livestock production system. Where people live with their livestock, they are also at higher risk of acquiring the disease [15]. The evidence for *Brucella* infections in cattle has been serologically evaluated by different authors [15, 16, 17, 18, 19]. According to some reports, *Brucella* seroprevalence is higher in the intensive farming systems than in extensive cattle rearing systems.

Recent reports by different reports [6, 20, 21, 22, 23] indicate that brucellosis is still a widespread disease, resulting in huge economic losses due to abortions. A limited number of studies have been conducted on brucellosis in a case-control approach. Moreover, almost all of the surveys were limited to the study of bovine brucellosis based on the cross-sectional study. A case-control study is paramount for *Brucella* organism assessment as a cause of abortion in cattle [24]. Hence, this study was carried out with the aim of investigating the risk factors of brucellosis in aborted cattle of Jimma zone, Ethiopia.

Results

From a total of 423 (141 cases and 282 controls) tested cattle 4.02% were positive for the anti-Brucella antibody by using CFT. A higher seroprevalence of brucellosis was observed in cases (6.38%) than controls (2.84%). Statistically significant ($p < 0.05$) differences in serostatus of the anti-Brucella antibody was observed among age categories. Relatively older animals were found to be more likely to be seropositive than their younger counterparts. Besides, variation in the seroprevalence of *Brucella* organism between the two breeds was statistically significant ($p < 0.05$). The local breed of cattle being almost four times (OR=4.04) more likely to harbor the anti-Brucella antibody compared to crossbred animals. Similarly, the variation in the serostatus of *Brucella* organism between pregnant and non-pregnant cows was statistically significant, where pregnant cows were three times (OR=3) more likely to harbor anti-Brucella antibody compared to non-pregnant ones. However, body condition, abortion period, retained fetal membrane and parity were not able to explain the seroprevalence distribution of the anti-Brucella antibody (Table 1).

Statistically significant variation ($p < 0.05$) was observed between Brucella serostatus and herd sizes. Cattle from large herd size category were almost eight times (OR= 8.29) more likely to be seropositive for anti-Brucella antibody than the cattle from the small herd size category. Similarly, a statistically significant difference in the serostatus of *Brucella* organism ($p < 0.05$) was observed in cattle herded with sheep and/goats; those having close contact with small ruminants had about four times (OR=4.42) more chance to be infected with *Brucella* organism than those with no contact. However, the study districts, agro-ecology, management system and introduction of the new animal were not significantly associated with anti-Brucella antibody distribution among cattle (Table 2).

No significant interactions ($p > 0.05$) between variables were detected. A Hosmer-Lemeshow goodness-of-fit value ($p = 0.94$), indicated that the model was fit the data. The model had the good predictive ability (ROC=0.82). The final multivariable logistic regression model showed that age, breed, herd size and species composition of domestic ruminants were independently associated ($p < 0.05$) with the seroprevalence of cattle (Table 3).

Discussion

A case-control study is paramount for the *Brucella* organism to investigate associated risk factors of brucellosis [24]. An overall of 4.02% seroprevalence of anti-Brucella antibody was recorded in the present study. A similar level of prevalence was reported by previous studies [27, 38], that reported a seroprevalence of 3.2% from the central and 4.8% from the southern Ethiopia. Likewise, other studies [40, 41]

Table 1
Univariable logistic regression analysis of host-related risk factors of cattle brucellosis in the study areas

Variables	Category	Cases	Controls	OR (95% CI)	*p*-value
Age					0.035
	<3 years (Ref)	11	18		
	3-6 years	67	137	3.92 (1.10-13.96)	0.035
	>6 years	63	127	5.92 (1.49-23.52)	0.012
Breed					
	Cross (Ref)	49	67		
	Local	92	215	4.04 (1.5-10.89)	0.003
BCS					0.269
	Poor (Ref)	21	33		
	Medium	91	173	1.84 (0.56-6.01)	0.313
	Good	29	76	4.12 (0.76-23.26)	0.109
Parity					0.216
	Nulliparous (Ref)	72	112		
	Monoparous	40	72	2.31 (0.63-8.47)	0.206
	Pluriparous	29	98	2.63 (0.72-9.62)	0.144
Pregnancy status					
	Non-Pregnant (Ref)	54	111		
	Pregnant	87	171	3.0 (1.09-8.28)	0.034
Abortion period					0.070
	No history (Ref)	0	282		
	After 5th month	44	0	1.26 (0.15-5.29)	0.832
	Before 5th month	97	0	0.33 (0.12-0.89)	0.029
Retained placenta					
	No (Ref)	75	219		
	Yes	66	63	0.478 (0.18-1.27)	0.138

OR: Odds Ratio; CI: Confidence Interval, Ref: Reference; BCS: Body condition score

reported 4.6% seroprevalence in selected regions of Ethiopia; [34] reported 3.1% in Jimma zone; [42] reported 4.3% in Adami Tulu and [43] reported 4.9% in northwest Ethiopia. Comparable prevalence was also reported by [44] 4.2% and 3.3% [45] in Eritrea and the Central African Republic, respectively. However, the seroprevalence report in this study is lower than some previous studies carried out in the country: 11.2% in East Shewa [46]; 6.1% in western Tigray [13]; 14.1% in Assela [47]; and 10.6% in Borana [21]. Similarly, higher seroprevalence was also reported in other African countries, For instance, 6.6% in Ghana [48], 41% in Togo [49], 6.6% in Chad [50] and 46.8% in Uganda [51]. On the other hand, the seroprevalence reported in the current study was higher than the 2.9% reported in central Ethiopia [52]; 1.7% in Sidama zone [17]; 2.6% in Arsi zone [23] and 1.4% in central Ethiopia [22, 53]. The variation in seroprevalence of brucellosis may be related to the prevalence of brucellosis that may vary based on the breed involved, management and environmental factors that influence the transmission rate of *Brucella* organism. This result is in agreement with a study [10] that reported that *Brucella* infection varies from country to country and

Table 2
Univariable logistic regression analysis of managemental and environmental-related risk factors of cattle brucellosis in the study areas

Variables	Category	Cases	Controls	OR (95% CI)	*p*-value
District					
	Limu Seka (Ref)	83	166		
	Chora Boter	58	116	1.71 (0.59-4.95)	0.321
Agro-ecology					
	Mid-altitude (Ref)	117	234		
	Lowland	24	48	3.39 (0.44-25.99)	0.240
Management system					
	Semi-intensive (Ref)	91	182		
	Extensive	50	100	1.61 (0.60-4.31)	0.345
Introduction of new animal					
	No (Ref)	68			
	Yes	73	146	2.71 (0.98-7.46)	0.054
Herd size					0.036
	Small (Ref)	60	120		
	Medium	34	68	2.18 (0.69-6.92)	0.187
	Large	47	94	8.29 (1.06-64.60)	0.044
Species composition					
	Only cattle (Ref)	8	16		
	Mixed with sheep and/ goat	133	266	4.42 (1.47-13.26)	0.008

OR: Odds Ratio; CI: Confidence Interval, Ref: Reference

also between regions even within a country.

In this study, an increase in age is associated with the increased risk of being *Brucella* seropositive; older animals (>6 years) were fourteen times (OR=14.16) more likely to be infected by brucellosis compared to their younger age groups. Similarly, several studies indicated age as one of the important risk factors influencing *Brucella* serostatus in cattle [12, 15, 16, 21, 32, 37, 54, 55, 56] in Ethiopia and elsewhere. This report is in line with the standard veterinary literature which supports younger animals tend to be more resistant to infection and being frequently infection-clear. Older animals are more susceptible to brucellosis than younger animals, which are due to sex hormones and erythritol that stimulate the growth and multiplication of bacteria [9].

There is an argument among different researchers on the issue of breed susceptibility to brucellosis. This study revealed that breed caused statistically significant variations in *Brucella* serostatus with the odd of the disease being five times (OR=5.36) higher in local than the crossbred breeds. The better management in the crossbred herds, intensive feeding that minimizes contacts between animals may be responsible for this difference. This finding is consistent with some previous studies in Ethiopia and elsewhere [12, 22, 43, 55, 57, 58] showing that the seropositivity for the anti-Brucella antibody was significantly associated with the breed in cattle. However, several studies [37, 39, 59, 60,] report that breed was not significantly associated with *Brucella* seropositivity in cattle in different parts of the country. Similarly, a few other studies [11, 61, 62] also reported no significant association between *Brucella* seropositivity and cattle breed in Zambia, Ni-

Table 3
Final multivariable logistic regression model of risk factors associated with cattle brucellosis in the study areas

Variables	Cases	Controls	Adjusted OR (95% CI)	*p*-value
Age				0.004
<3 years (Ref)	11	18		
3-6 years	67	137	6.43 (1.46-12.34)	0.014
>6 years	63	127	14.16 (2.91-28.84)	0.001
Breed				
Cross (Ref)	49	67		
Local	92	215	5.36 (1.76-11.33)	0.003
Herd size				0.037
Small (Ref)	60	120		
Medium	34	68	2.77 (0.62-2.93)	0.109
Large	47	94	11.82 (1.31-16.17)	0.024
Species composition				
Only cattle (Ref)	8	16		
Mixed with sheep and/ goat	133	266	5.10 (1.49-13.43)	0.009

OR: Odds Ratio; CI: Confidence Interval, Ref: Reference

geria, and Malaysia, respectively. This variation could be due to the difference in environmental factors and management systems.

In the present study, statistically significant variation has been observed in the seroprevalence of anti-Brucella antibody between different herd sizes; larger herd sizes were almost twelve times (OR=11.82) more likely to be seropositive. Herd size has previously been reported in Eritrea as an important determinant for transmission of *Brucella* organism between susceptible and infected animals [44] and thus; larger herds were more likely to have at least one positive animal than smaller herds [63]. Several authors in Ethiopia and Zimbabwe also reported that large herd size enhances the exposure to and maintenance of *Brucella* organisms following abortions through increased contact at common feeding and watering points [12, 16, 17, 18, 64]. However, contrary to this another study [37] reported that the risk of seropositivity was independent of herd size in the central Ethiopia. The observed variation could be attributed to various factors including agro-ecology and management system.

In this study, cows from households herding cattle together with goats and/ sheep had five times (OR=5.10) more odds of brucellosis than those kept without other species. Herding of these animals together increases the chance of cross-species transmission of *Brucella* organisms. *Brucella* organism is not strictly host-specific; *Brucella melitensis* has been isolated from cattle [65] and thus, herding together might have increased the spillover of the pathogen from small ruminants to cattle. Moreover, herding more cattle at one farm may increase animal density and chance of contact among animals, as a result, facilitating exposure to *Brucella* species and increasing the chance of acquiring the disease [66]. This finding is in line with the previous study [21], that reported the mixing of sheep and/ goats with cattle increased risk of *Brucella* seropositivity in cattle in Borana zone, Ethiopia. Moreover, other reports from Eritrea [44], Malaysia [62] and Jordan [63] also confirmed that mixed farming especially raising sheep and/ goats along with cattle was a risk for *Brucella* spread among different animal species. This is different from the findings of another study [67], that reported that keeping sheep and/ goats with cattle is not significantly associated with *Brucella* seropositivity in Sudan. This variation could be due to the difference in environmental factors, breed of animals, and management system.

In conclusion, the present study shows that cases have higher *Brucella* seropositive status than control cattle groups. Higher *Brucella* seropositivity was recorded in this study. This indicates that brucellosis causes huge economic losses and serious public health

problem. The present study identified that age, breed, herd size, and species compositions as risk factors for *Brucella* seropositivity in cattle. Hence, different livestock species need to be kept and maintained separately to reduce the risk of transmission of *Brucella* among them. It is also important to conduct applicable control methods and increasing public awareness of the zoonotic transmission of brucellosis. Moreover, further study should be carried out to identify the specific causes of abortion in cattle for the preparation of the appropriate vaccine.

Material and methods

Study areas

The study was conducted from October 2016 to October 2017 in selected districts of Jimma zone. These districts are one of the potential areas for cattle production in the zone. Limu Seka district is situated 109 km from Jimma town. The district is located at an altitude of 1400-2200 meter above sea level, 09°29' North latitude and 37°26' East longitudes. The agroecology is characterized by 13% highland and 55% mid-highland and 32% lowland. The average temperature varies from a minimum of 15.1°C to a maximum 31°C. There are two distinct seasons in Limu Seka: the rainy season (from late March to October), and the dry season (November to early March). Limu Seka district has 295,627 cattle, 104,892 sheep, 89,079 goats and 134,370 human populations. Chora Boter district is located 112 km from Jimma town. The district is located at 9°-10°24' North latitude and 37°56'-40°35' East longitude with an altitude range of 1100-2200 meter above sea level. The agroecology is characterized by 25% highland, 73.5% mid-highland and 2.3% lowland. The annual average temperature ranges from 18.3°C to 26.7°C. Similar to the Limu Seka district, the district has two seasons. The rainfall is often more than 1,800-2,200 mm per annum. Chora Boter district has 228,846 cattle, 47,854 sheep, 68,037 goats and 215,348 human populations. There are two management systems in the area: these are extensive (crop-livestock production) systems and semi-intensive (urban production). Local cattle are the dominant breed in the area and crossbred Holstein- Friesian also present (Figure 1).

Study population, design and methods

Target populations were female cattle in the selected districts of Jimma zone. The study population was breeding cattle in selected peasant associations of the study districts. Animals in this study were female cattle from herd having three and above cows and/or heifers with a history of abortion. Case-Control study design was used, where cows or heifers that had experienced abortion were defined as cases. Controls were cows or heifers from the same herd but had no record of abortion with the age of two years and above. Abortion was the loss of pregnancy from 42 to 260 days of gestation [1] for this study. Jimma zone was selected purposively based on the dominant of cattle population, while the peasant associations, village, and herd were selected randomly. A simple sampling method was used to select a sample of animals. Cattle involved in this study had no vaccination against brucellosis.

Sampling procedure and sample size determination

Limu Seka and Chora Boter districts were selected purposively based on the history of abortion. A total of ten peasant associations were included from these districts using a random sampling technique, where six peasant associations were from Limu Seka and four of them from Chora Boter. Cows/heifers with a history of abortion in the herd were selected purposely based on districts' veterinary clinic case book and the owners' information. Before the selection of herds with the history of abortion, the availability of cases were checked. To incorporate more herds (clusters),

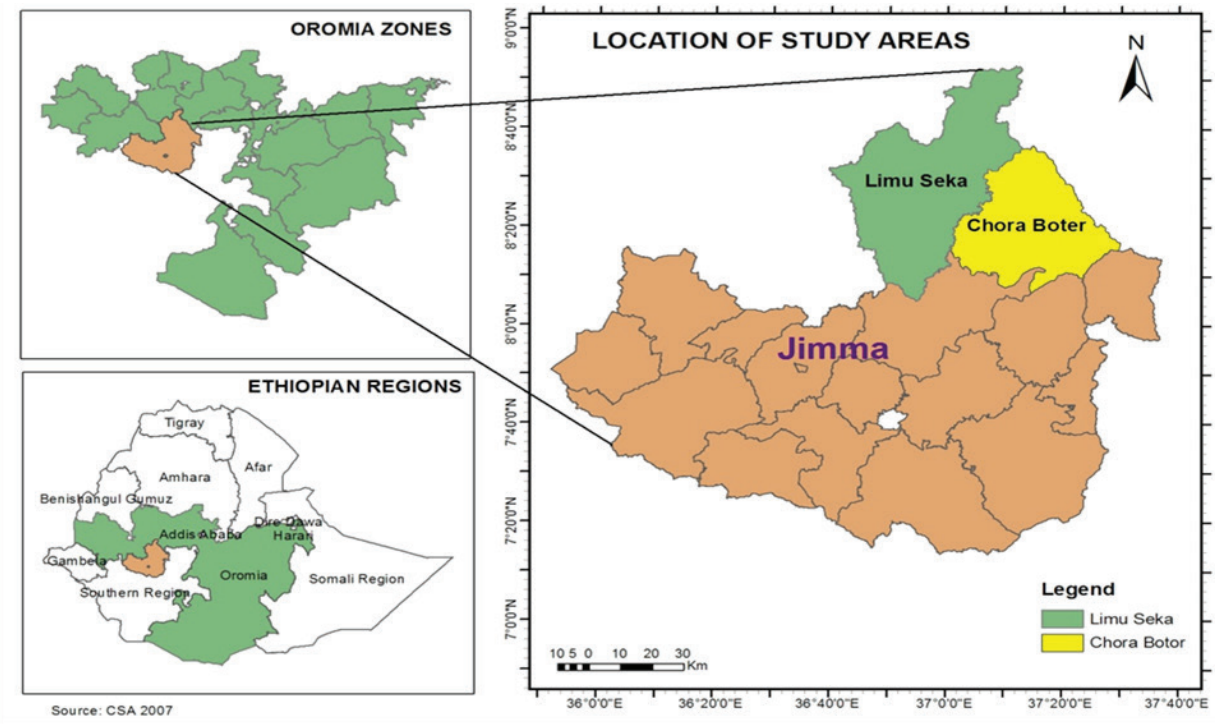

Figure 1
Map of the study areas (Limu Seka and Chora Boter districts).

the number of cases per herd was limited to a maximum of two and four control per herd. The necessary minimum sample size was calculated using [25] base on case-control study design with a predetermined odds ratio (OR) of 3, an expected prevalence of exposure in control groups of 10%, a desired level of 95% confidence, 5% precision, and a power of 80% [26, 27], thus leading to a sample size of 97 cases. With two controls selected per case, the number of controls should have been 194. To adjust the difference among the clusters, the sample size was multiplied by the design effect (D) by using the formula D= ρ (n-1) + 1, where n is average number of dairy cattle in cluster (6), and intra-cluster correlation coefficient of ρ = 0.09 has been reported for *B. abortus* in cattle [28]. The design effect (D) was 1.45 and increasing the power by using two controls per case. Thus, a minimum of 141 cases and 282 controls were selected to be enrolled in this study. Eventually, a total of 423 cows were involved in the study. Selecting sample animals, a sampling frame of the herd with abortion was prepared in collaboration with district veterinary departments and a total of 118 herds were chosen at random. In each herd where one or two cases were found, these were selected and controls were chosen at random using a lottery method. On the other hand, a random sampling method was used where large cases and controls were available in the herd.

Data collection

Information related to district, agro-ecology, age, body condition, breed, parity, pregnancy status, history of retained fetal membrane, abortion period, herd size, management system, introduction of new animal and species composition (mixed of cattle with sheep and/ goats) were gathered using a separate format prepared for this purpose. Classification of management systems (extensive and semi-intensive) was done based on the criteria adopted by [29]. Body condition score was based on the criteria adopted by [30] and for all cows, under the study, their body condition grouped into three groups (poor, medium and good). Age of animals was categorized into <3, 3-6 and >6 years and groups were chosen because optimal age at first calving cattle reared under tropical conditions was estimated to be 2-3 years [31]. Herd size was categorized into small (3-5 heads of cattle), medium (6-10 heads of cattle) and large (>10 heads of cattle). Those cattle that kept in the same barns grouped and considered as one herd [16, 32]. Parity number was categorized as nulliparous (zero parity), monoparous (parity one) and pluriparous (≥ two parities) [33, 34].

Blood sample collection

Approximately 10 milliliters of blood samples were collected from the jugular vein of each animal, using sterile needles and plain vacutainer tubes. The identification of each animal was labeled on the corresponding vacutainer tubes and blood samples were allowed to stand overnight (12 hours) at room temperature to obtain the serum. The animals' identification codes were transferred to the cryovials to which the serum was decanted and serum samples were kept at -20°C [35] in Jimma University microbiology laboratory until they transported to National Veterinary Institute, Debrezeite using icebox for serological analysis.

Rose Bengal Plate Test

The serum samples were screened by using Rose Bengal Plate Test (RBPT) (KT153NB, UK) for the presence of *Brucella* agglutinins according to the previously published procedure [35]. Serum samples and antigens when taken out from the refrigerator, will be kept at room temperature for half an hour and processed following the recommended procedure. A total of 30 microliters of serum sample was dispensed onto the plate and 30 microliters of RBPT antigen were dropped on the slide with sera. The interpretation of both positive and negative control results was done according to the degree of agglutination and the reaction was read in a good light source or by a magnifying glass when micro agglutination was suspected. The RBPT results were interpreted 0, +, ++ and +++ as has been described by [26], where 0 indicates no agglutination, + indicates barely visible agglutination (using magnifying glasses), ++ indicates fine agglutination and +++ indicates coarse clumping. Those serums identified with no agglutination (0) were regarded as negative, while those with +, ++ and +++ were considered as positive.

Complement fixation test

All RBPT positive sera were further tested using a complement fixation test (CFT) using standard *B. abortus* antigen S99 and control sera (positive and negative) (KT15 3NB, United Kingdom). The antigen dilution was standardized at 1:10. Two-fold dilutions (1:5, 1:10, 1:20 and 1:40) of test sera were ready in standard 96-well U-bottom microtiter plates before adding *Brucella* antigen, guinea pigs complement and 3% sensitized sheep red blood cells. The preparation of the reagent was evaluated by titration and performed according to the recommended protocols by [7]. The plates were incubated at 37°C for thirty-minute with agitations and results were read after the plates have been centrifuged at 2500 rpm for five minutes at 4°C. Sera with a strong reaction, more than 75% fixation of complement (3+) at 1:5 dilution or at least with 50% complement fixation (2+) at 1:10 dilution and above were considered as positive and lack of fixation/ complete hemolysis was considered as negative [35]. An animal was considered positive if tested seropositive on both RBPT and CFT in serial interpretation. Both the Rose Bengal plate test and the Complement fixation test were done in the National Veterinary Institute (NVI). The combination of RBPT and CFT in serial most widely used is commonly recommended to maximize the specificity of the test result by ruling out false-positive serological cross-reactions [26].

Data management and analysis

The collected data were stored in Microsoft Excel for Windows 2010 and then transferred to SPSS version 20.0 (IBM SPSS, 2011) for analysis. The seroprevalence of brucellosis was calculated by dividing the number of seropositive samples to the total of cattle samples. The association between brucellosis and associated risk factors were analyzed using logistic regression model. Risk factors associated with brucellosis were identified by using a multivariable logistic regression model and the strength of their association was assessed using adjusted odds ratios (OR). Variable with a *p*-value less than or equal to 0.25 in the univariable analysis were involved in the multivariable logistic model. The backward elimination procedure was used for a further selection of variables. The variables were tested for interaction effect using cross-product terms and for multiple-collinearity using the collinear matrix index before building the final model [36]. Hosmer-Lemeshow test was used to evaluate the validity of the model. Similarly, the predictive ability of the model was assessed using the ROC curve. Confidence level (CL) was at 95% and $p \leq 0.05$ were set for significance for all analyses.

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Author Contributions

D.T. contributed to sample collection, laboratory tests, data analysis and drafting the manuscript. B.D. and F.B. contributed to the main design of the study, and reviewed and edited the manuscript. All authors approved the final version of the manuscript for publication.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Comparing the protective effects of L-carnitine and *Silybum marianum* aqueous extract after diazinon-induced hepatotoxicity in male rat liver

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ABSTRACT

Diazinon (DZN), as an organophosphorus pesticide, induces oxidative stress leading to the generation of free radicals, and causes some pathological changes in the body. The purpose of this study was to assess the protective effects of L-carnitine (LC) and *Silybum marianum* aqueous extract (SMAE) against DZN-induced hepatotoxicity in male rat liver. Rats were assigned in 9 groups and were subjected to different combinations of DZN, SMAE, and LC. Thirty days after the treatment by oral gavage, blood samples were taken and serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), Albumin (Alb) and total protein (TP) were measured using photometric method. The liver samples were also evaluated histopathologically. The DZN treatment significantly increased the AST, ALT, ALP and GGT levels ($p < 0.05$) and conversely decreased the Alb and TP levels ($p < 0.05$). Moreover, administration of the DZN resulted in lymphocytic inflammation, congestion, hepatocytes apoptosis, and lesser sinusoidal space. However, administration of SMAE and LC along with DZN treatment stabilized the ALT, ALP, GGT, Alb and TP levels ($p > 0.05$), but increased the AST levels significantly compared to the control ($p < 0.05$). Besides that, lesser structural alterations and improvement in the liver tissue were observed. These findings suggest that co-administration of SMAE and LC could reduce DZN-induced hepatic tissue damages and improve the hepatic biochemical parameters in male rats.

Keywords

Diazinon, L-carnitine, Silybummarianum, Hepatotoxicity, Rat

Abbreviations

DZN: Diazinon
OP: Organophosphorus pesticide
LC: L-carnitine
SAME: *Silybum Marianum* Aqueous Extract
SM: *Silybum Marianum*
AST: Aspartate aminotransferase
ALT: Alanine aminotransferase
ALP: Alkaline phosphatase
GGT: Gamma-glutamyl transferase
Alb: Albumin
TP: Total protein

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Introduction

Liver is the largest gland in the body which performs a variety of functions, including protein synthesis, storing required substances, metabolic functions, disinfecting harmful substances, producing and secreting bile as well as controlling metabolism [1]. Organophosphorus pesticide (OP) refers to various toxic compounds that contain phosphorus in their basic structure. They have been introduced to the world market since 1960s. Since they were more potent than other toxins such as organochlorine, OP compounds replaced other toxins rapidly. In 1970, due to their environmental and food chain contamination they were only used in certain cases in developed countries, however, they are still used in some countries [2].

One of the most important OPs used to control insects in agriculture is DZN. This compound is easily and quickly (within a few hours) absorbed by the intestine [3,4]. This compound inhibits acetylcholine esterase through the phosphorylation of amino acid serine at its active site, resulting in the accumulation of acetylcholine in cholinergic synapses, and causing cholinergic seizure and brain injury and death in acute cases [5,6]. DZN is also absorbed through skin and respiratory tract, and rapidly metabolized to diazoxide in the liver. Most OP compounds are converted to active toxic metabolites in the liver by the cytochrome P450 system through oxidative desulphurization [7]. It should be noted that the side effects of these compounds depend on the type of toxin, dose, duration of exposure and also the tissue. The main mechanism of OP action, especially DZN, is inhibiting acetylcholine esterase [8]. They are alkylated agents that react with cellular macromolecules, such as proteins, nucleic acids and lipids, and alter their functions [9]. Some researchers believe that DZN induces oxidative stress by producing free radicals and reactive oxygen, and induces cell death in organisms by increasing lipid peroxidation [10]. Indeed, exposure to DZN causes severe histopathologic damages in the kidney and liver [11].

Antioxidant compounds play an important role in preventing damages and pathological changes caused by free radicals. For example, LC prevents oxidative stress and regulates nitric acid, cellular respiration, and the activity of enzymes involved in oxidative stress [12,13]. The antioxidant system consists of three enzymes: glutathione peroxidase, catalase and superoxide dismutase. As an antioxidant compound, LC can protect these enzymes against oxidative damage; it is also very effective in modulating age-related changes [14]. Food sources of amino acid carnitine are very important. Approximately 75% of total carnitine is obtained from carnitine, lysine, and methionine food sources [15]. The condition of carnitine in the human body depends on the body composition, sex and diet. There are two sources for these amino acids: (1) diet and (2) degradation of endogenous proteins. Micronutrients such as iron, vitamin C, pyridoxine and niacin are essential for the synthesis of carnitine [16].

The plant *Silybum marianum* (SM) is an annual or biennial plant of the *Asteraceae* family [17]. SM has global vegetation and is native to Iran as well [18]. SM contains silybin, isosilybin, silydianin and taxifolin, which are collectively called flavonoids silymarin. Silymarin is found in all parts of the plant and is considered as the main and effective compound of SM. The main pharmacological properties of this plant include: antioxidant, anti-inflammatory and anti-cancer effects and also protecting hepatic cells against many liver toxins [19,20].

Since LC and SM show antioxidant and anti-inflammatory effects and are used in the treatment of liver disorders, in this study, we tried to investigate their possible hepatoprotective effects in DZN-induced hepatic damage in male rats.

Results

Biochemical analysis findings

Table 1 and 2 represent the mean and standard deviation (SD) of the serum levels of ALT, AST, ALP, and GGT as well as Alb and TP among different groups, respectively. The findings of this study showed that there was no significant difference in the serum levels of ALT, AST, ALP, GGT, Alb and TP between the control and sham groups ($p > 0.05$). Compared to the control and sham groups, the serum levels of ALT, AST, ALP, and GGT increased significantly ($p < 0.05$) in the DZN15 group, while the serum levels of Alb and TP decreased significantly ($p < 0.05$).

Statistical comparison of the results showed that there were no significant differences in the serum levels of ALT, AST, ALP, GGT, Alb and TP in the SMAE100, LC300 and SMAE100 + LC300 groups, when compared to the control and sham groups ($p > 0.05$).

In the DZN15+MS100 and DZN15+LC300 groups, the serum levels of ALT, AST, ALP, and GGT increased significantly compared to the control and sham groups ($p < 0.05$); however, they showed a significant decrease compared to the DZN15 group ($p < 0.05$). In addition, the serum levels of Alb and TP decreased significantly in the DZN15 + MS100 and DZN15 + LC300 groups compared to the control and sham groups ($p < 0.05$); while, showed a significant increase compared to the DZN15 group ($p < 0.05$).

In the DZN15 + SMAE100 + LC300 group, the se-

Table 1
Comparison of serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) in different groups.

Groups	AST (u/l)	ALT (u/l)	ALP (u/l)	GGT (u/l)
Control	359.87 ± 10.46	82.37 ± 5.18	777.62 ± 16.26	5.37 ± 0.406
Sham	365.12 ± 09.49	84.87 ± 4.51	769.75 ± 13.62	5.27 ± 0.541
DZN15	782.25 ± 11.81 [a]	180.25 ± 9.06 [a]	1278.62 ± 37.30 [a]	11.43 ± 0.462 [a]
SMAE100	362.25 ± 10.12 [b]	84.62 ± 7.81 [b]	779.00 ± 18.60 [b]	5.26 ± 0.434 [b]
LC300	356.00 ± 09.05 [b]	85.42 ± 4.57 [b]	773.00 ± 16.69 [b]	4.87 ± 0.281 [b]
SMAE100 + LC300	352.25 ± 08.27 [b]	81.00 ± 4.24 [b]	769.75 ± 14.95 [b]	4.91 ± 0.285 [b]
DZN15 + LC300	533.00 ± 07.65 [a,b]	115.125 ± 5.56 [a,b]	1001 ± 55.79 [a,b]	7.68 ± 0.318 [a,b]
DZN15 + SMAE100	513.28 ± 07.52 [a,b]	105.42 ± 10.57 [a,b]	949.00 ± 73.07 [a,b]	6.87 ± 1.17 [a,b]
DZN15+ SMAE100 + LC300	411.87 ± 13.95 [a,b]	90.25 ± 4.13 [b]	804.12 ± 19.88 [b]	5.22 ± 0.310 [b]

Data is shown as mean ± SD.
a, $p < 0.05$ as compared to the control and sham groups
b, $p < 0.05$ as compared to the DZN15 group

Table 2
Comparison of serum levels of Albumin (Alb) and Total protein (TP) in different groups.

Groups	Alb (g/dl)	TP (g/dl)
Control	3.55 ± 0.244	6.58 ± 0.473
Sham	3.53 ± 0.277	6.52 ± 0.337
DZN15	1.75 ± 1.92 [a]	3.85 ± 0.272 [a]
SMAE100	3.60 ± 0.261 [b]	6.28 ± 0.485 [b]
LC300	3.52 ± 0.269 [b]	6.20 ± 0.258 [b]
SMAE100 + LC300	3.97 ± 0.361 [b]	6.00 ± 0.392 [b]
DZN15 + LC300	2.68 ± 0.247 [a,b]	5.30 ± 0.272 [a,b]
DZN15 + SMAE100	3.24 ± 0.403 [a,b]	5.23 ± 0.297 [a,b]
DZN15 + SMAE100 + LC300	3.47 ± 0.265 [b]	6.67 ± 0.310 [b]

Data is shown as mean ± SD.
a, $p < 0.05$ as compared to the control and sham groups
b, $p < 0.05$ as compared to the DZN15 group

rum levels of ALT, AST, ALP, and GGT decreased significantly compared to the DZN group ($p < 0.05$) but the serum levels of Alb and TP increased significantly ($p < 0.05$). Although, there was no significant difference in the serum levels of ALT, ALP, GGT, Alb and TP ($p > 0.05$), the serum levels of AST increased significantly in the DZN15 + SMAE100 + LC300 group compared to the control and sham groups ($p < 0.05$).

Histopathological findings

Histopathological findings on the liver tissues indicated that the liver parenchymal cells, sinusoid spaces and central vein in the control and sham groups were completely normal and were not damaged (Figure 1A and 1B). In the DZN15 group, lymphocytic inflammation, congestion, hepatocyte apoptosis, and lesser sinusoidal space were observed (Figure 1C). There were no signs of liver damage in the LC300, SMAE100, and SMAE100 + LC300 groups (Figure 1 D, 1E and 1F). In the DZN15 + LC300 and DZN15 + SMAE100 groups, lymphocytic inflammation and vacuolization of cytoplasm were detected (Figure 1G and 1H). Histological cross-sectional study in the DZN15 + SMAE100 + LC300 group revealed lesser pathological changes and improvement in the liver tissue compared to the DZN group (Figure 1I).

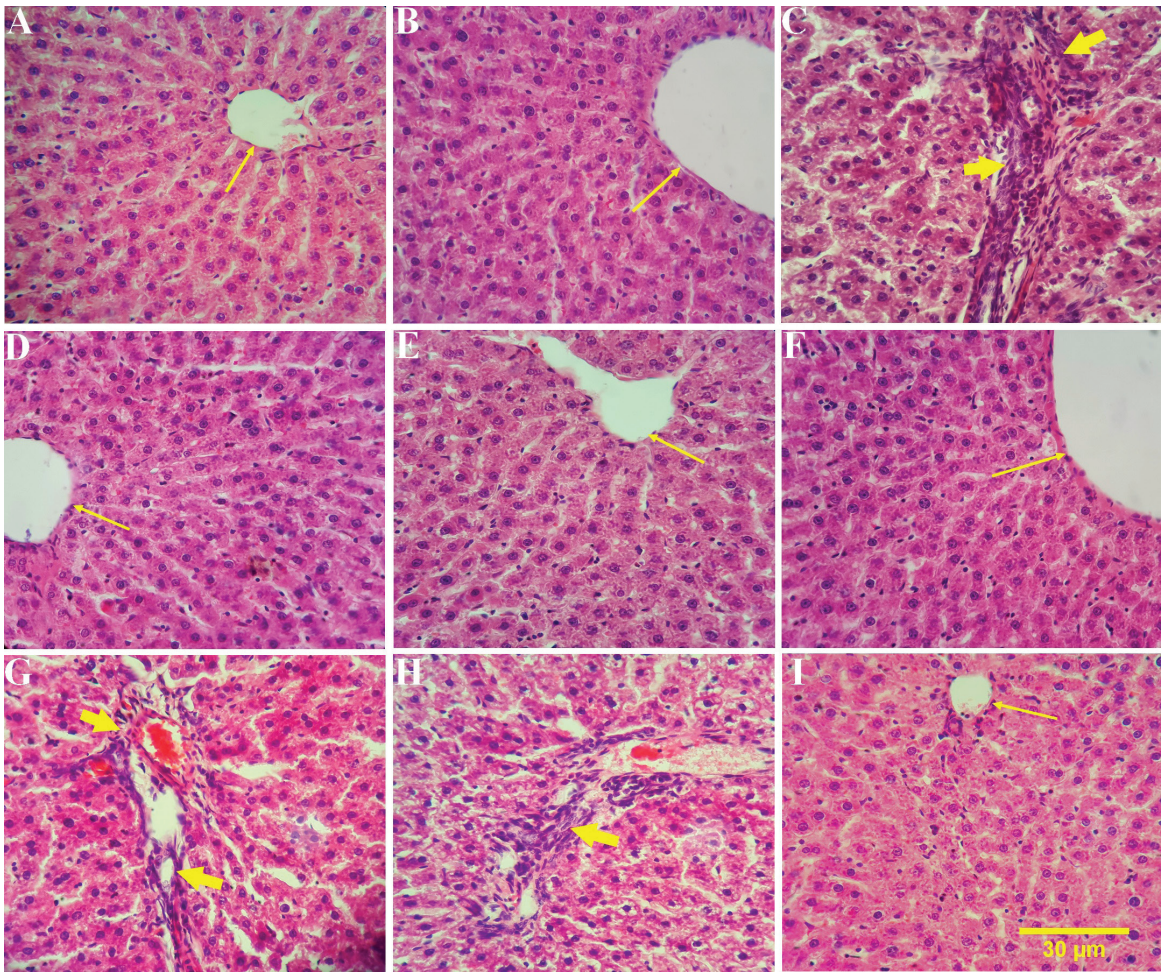

Figure 1
Histopatological analysis of liver in different groups. H&E 100× (A-I). A) Control, B) Sham, C) DZN15, D) SMAE100, E) LC300, F) SMAE100+LC300, G) DZN15+ LC300, H) DZN15+SMAE100, I) DZN15+SMAE100+LC300.

Discussion

In the present experimental study, the effects of LC, SMAE and DZN toxin on the serum levels of hepatic enzymes (ALP, ALT, AST and GGT), Alb and TP in adult male rats were investigated. Since these hepatic enzymes are intracellular, and enter bloodstream in cases where cell damage occurs, it can be concluded that DZN is able to damage hepatocytes.

In the present study, the elevated level of ALP in the DZN15 group is possibly the result of the cholestasis, and the increase in the levels of ALT and AST in this group is likely to result from liver cell necrosis. Similarly, it has been shown that the intake and absorption of DZN by the liver increases the serum levels of ALT, ALP and AST in mice [21]. Also, in their experiments on rabbits, Solati et al., reported that DZN increases activities of ALT, ALP and AST [22], which is consistent with the results of this study. Gokcimen et al., reported that DZN caused pathological changes including hepatocyte necrosis and infiltration of inflammatory cells due to the activity of detoxification of the liver and the production of high levels of free radicals in the liver cells in rats [23]. OP damage the cells and tissues of the body by increasing lipid peroxidation, cell apoptosis and free radical production as well as inhibition of the activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase [24,25].

In the SMAE100, LC300 and SMAE100+LC300 groups, administrating SMAE and LC alone as well as co-administration of SAME+LC did not make a significant difference in the serum levels of AST, ALT, ALP, GGT, Alb and TP in comparison with the control and sham groups. However, in all three groups compared to the DZN group, the serum levels of liver enzymes decreased significantly, while the serum levels of Alb and TP increased significantly. In accordance with the results of biochemical parameters, histopathologic study in all three groups indicated that the liver tissue structure was completely normal and similar to the control and sham groups. It has been reported that the use of natural antioxidant compounds can play an important role in improving and decreasing oxidative stress damages. The safety of synthetic antioxidants has always been questioned, therefore, using natural

plant sources has been considered with regard to active antioxidant compounds in the recent years (26). SM has a wide range of biochemical effects on various tissues of the body (27). This plant inhibits metabolic disorders by increasing cellular glutathione level and due to the fact that it is a potent antioxidant, it can prevent metabolic disturbances and apoptosis by removing free oxygen radicals (28,29).

It has been shown that prescribing LC at a dose of 100 mg/kg can increase glutathione levels (30). LC stimulates the complex of pyruvate dehydrogenase and increases the entry of pyruvate into the beta oxidation pathway, which causes more use of oxygen and lipids oxidation. It also reduces the production of free radicals by reducing the production of Acetyl-CoA and helps to repair the oxidative damage of the phospholipid membrane and ultimately to prevent apoptosis (31).

Administrating LC and SMAE in the DZN15 + LC300 and DZN15 + SMAE100 groups showed that compared to the control and DZN15 groups, the serum levels of AST, ALT, ALP and GGT significantly increased and decreased, respectively, while the serum levels of Alb and TP decreased and increased significantly, respectively. Also, according to histopathologic results and the significant changes in biochemical parameters, lymphocytic inflammation and vacuolization of cytoplasmic space of hepatocytes were observed. The results of this study indicate that the administration of LC and SMAE alone in the doses used can improve the relative biochemical parameters and histopathologic changes compared to the DZN15 group, however, it seems that co-administration of LC and SMAE would be effective in reducing further hepatotoxicity effects induced by DZN.

According to the results obtained for DZN15+SMAE100+LC300 group, simultaneous administration of SMAE and LC reduces the serum levels of ALT, ALP and AST relative to the DZN15 group. This means that the simultaneous use of both the extract and the drug has an effective protective effect on the hepatocyte damages caused by DZN. Polyphenolic compounds in the SMAE and LC can protect the liver cells against DZN toxin through different mechanisms. They may preserve the cell membrane stability through preventing the oxidation of membrane lipids by the active metabolites produced from DZN intake. They can also repair cell damages by stimulating protein synthesis in damaged cells. Phenolic compounds may also reduce the damage caused by DZN through stimulating detoxification systems as well as increasing the glutathione-reduction capacity of the hepatocytes [27,32,33]. Of course, these effects can be more noticeable in the long term consumpsion of SMAE and LC.

It can be argued that SMAE prevents the progression of liver damage and inhibits liver cirrhosis by its numerous properties such as antioxidant, anti-lipid peroxidase, anti-fibrotic, anti-inflammatory, immune-regulation and liver cell regeneration. The significantly decreased AST and ALT levels in the DZN15+SMAE100 group confirms that the compounds present in SMAE can protect outer membrane of the hepatocytes, preventing penetration of toxic substances as well as increasing the ribosomal protein synthesis and thus, improving liver function [34].

In an in vitro study, Gulchin showed that LC has an antioxidant effect against free radicals of superoxide and hydrogen peroxide [35]. Hence, as an antioxidant compound capable of destroying free radicals, LC is able to reduce the damage caused by DZN in the liver tissue.

According to our results, the serum levels of Alb and TP significantly declined in the DZN15 groups. Alb is one of the most important circulating proteins that accounts for more than half of the plasma protein. It is made by the liver and secreted in the bloodstream. Blood Alb level is a sensitive and valuable parameter of liver function and its decline is a sign of liver failure [36]. A recent study by Yehia et al., on rabbits poisoned with DZN reports a decrease in Alb and TP levels; this decline was considered to be the result of an increase in proteolytic activity due to post-poisoning stress [37].

Since the levels of Alb and TP significantly declined in the DZN15 group while their levels increased significantly in the groups receiving mixture of DZN, LC and or SMAE, it can be argued that DZN toxin damages hepatic tissues leading to failure in producing enough Alb, but when the SMAE and LC are used (especially in the DZN15+SMAE100+LC300 group) the liver functions are restored. In other words, the concomitant use of the toxin, extract or the drug may have effective protective influences on the liver cells against the damage caused by DZN. These beneficial effects may essentially be the result of antioxidant properties of phenolic compounds present in SM, which allows them to act as reducing agents (hydrogen donor and oxygen inactivator). In general, the results of this study showed that the combination of SMAE and LC can have beneficial effects on restoring serum levels of hepatic enzymes and proteins like Alb in rats intoxicated by DZN. This combination may have antioxidant properties capable of eliminating free radicals leading to a reduction in DZN-induced hepatic injuries that provides a suitable therapeutic approach to use in such toxin poisoning.

Material and methods

Animals

Seventy-two adult male Wistar rats weighing 220 ± 20 g and 2 months old were provided from the animal house of Kazerun Islamic Azad University and were kept at the same place. The animals were supplied with water and food ad libitum. During the study, animals were exposed to standard conditions at 22 ± 2 ºC, 12-hour light and 12-hour darkness cycles and 70% humidity in polycarbonate cages. The protocol of this study was approved by the Ethics Committee of Islamic Azad University of Kazerun, Iran, in relation to working with laboratory animal care (No. IR.Kiau 15230509971001).

Medications

LC was purchased from Merck Company (Germany) and technical DZN with 95 percent purity was obtained from Sam Gol Company (Iran). Normal saline was used to prepare LC solution.

Preparing SMAE

The aerial part of the SM contains the stem and seeds dried and then powdered. 100 g of the powder was added to 500 ml of distilled water and mixed well, and then kept at room temperature for 24 hours. The mixture was mixed using magnetic heating stirrer at 60 °C for 1 hour. The extract was centrifugated for 20 minutes at 10,000 rpm and then filtered. The extract was stored in the refrigerator until it was used [38].

Study design

The animals were assigned in 9 groups of 8 each including: the control group (left untreated and only received water and food), the sham group (received normal food, water and 1 ml distilled water as the drug solvent), the DZN group (received 15 mg/kg DZN), the SMAE100 group (received 100 mg/kg SM aqueous extract), the LC300 group (received 300 mg/kg LC), the SMAE100+LC300 group (received 100 mg/kg SM aqueous extract and 300 mg/kg LC in the afternoon), the DZN+LC300 group (received 15 mg/kg DZN in the morning and 300 mg/kg LC in the afternoon), the DZN+SMAE100 group (received 15 mg/kg DZN in the morning and 100 mg/kg SM aqueous extract in the afternoon), and the DZN+SMAE100+LC300 group (received 15 mg/kg DZN in the morning and 100 mg/kg SM aqueous extract as well as 300 mg/kg LC in the afternoon). The extract and the drugs were administered in all groups by oral gavage for 30 days. The acute oral LD50 (the dose to kill half the population of laboratory animals) of DZN have been determined to be 1250 mg/kg body weight for the rats [39,40]. The basis for selecting the dose of DZN and LC was based on the previous studies (40,41).

Biochemical analysis

At the end of the treatment period, animals were anesthetized with diethyl ether (Merck, Germany), and blood samples were taken from their left heart ventricle using a 5 ml sterile syringe. Blood samples were transferred into the test tubes without anticoagulant, and were centrifugated at 5000 rpm for 15 minutes. The supernatant (serum) was isolated by sampler, transferred to new tubes, covered by Parafilm, and stored at -20 °C to be tested later. The serum levels of AST, ALT, ALP, GGT, Alb and TP were evaluated spectrophotometrically (Pars kits, Pars Co., Iran) using the Technicon RA-1000 machine (USA).

Histopathological analysis

After blood sampling, and euthanasia, the animal's abdominal cavity was opened and the livers of all rats were removed. The tissue samples were washed with physiological normal saline and fixed in 10% formalin buffer solution. After the dehydration in alcohol with increasing concentrations of 60% to absolute, they became transparent using xylene and then were blocked in paraffin. 10 sections of 5-micron thickness of each liver tissue were stained by H & E method and eventually, the tissue changes were studied using a light microscope. In each section, five different areas were randomly investigated, and the number of lymphocytes, the levels of apoptosis, congestion, sinusoidal spaces, and vacuolization of cytoplasm were studied.

Statistical analysis

The results were analyzed using SPSS software version 20 (SPSS Inc., Chicago, IL, USA). The results normalization were confirmed using the Kolmogorov-Smirnov test. The significance level was defined as $p < 0.05$, and was applied to examine the significant difference between the experimental and control groups by one-way ANOVA and post-hoc Tukey's tests. The results are shown as mean ± standard deviation (SD) in the tables.

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Author Contributions

Designed the experiments, performed statistical analysis and revised the manuscript: M.S., M.M. Performed histopathological analysis and wrote the manuscript: F.M. All authors approved the final version of the manuscript.

Conflict of Interest

None declared.

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RESEARCH ARTICLE

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Effects of dietary β-1,3-glucan and host gut-derived probiotic bacteria on hemato-immunological indices and gut microbiota of juvenile rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The effects of indigenous probiotics *Lactobacillus plantarum* and *Lactobacillus pentosus* alone, and in combination with β-1,3-glucan in juvenile rainbow trout (*Oncorhynchus mykiss*) were investigated. Eight groups were defined: control (G1), 1% β-1,3-glucan (G2), *L. plantarum* (G3), *L. pentosus* (G4), *L. plantarum* + *L. pentosus* (G5), *L. plantarum* with 1% β-1,3-glucan (G6), *L. pentosus* with 1% β-1,3-glucan (G7) and *L. plantarum* + *L. pentosus* with 1% β-1,3-glucan (G8). After eight weeks, the innate immune responses were elevated in all treated groups; however, synergistic effects were observed for anti-trypsin, bactericidal activity and respiratory burst activity in groups 7 and 8. Although the other immune responses were higher in treated groups, they did not make statistically significant differences. Checking microbiota showed that β-1,3-glucan improved conditions of indigenous probiotics. The diet 8 caused significant alterations in the intestinal microbiota by significantly decreasing the proportion of total count bacteria to lactic acid bacteria, which were demonstrated by reducing the total number of bacteria in Group 8 compared to the control group.

Keywords

Oncorhynchus mykiss, host gut-derived probiotics, immune response, synbiotic, β-1,3-glucan, intestinal microbiota

Abbreviations

RBC: Red Blood Cell
WBC:White Blood Cell
Hb: Hemoglobin
NBT: Nitro Blue Tetrazolium

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Introduction

In recent years, cold-water fish culture has developed into a very thriving industry, due to its priority in various aspects, including its meat. This development has led to overcrowding of trout farming, combined with the increase in intensive production strategies at higher densities. Different sources of stress such as high stocking densities and manipulations, adversely affect the immune system, leading to the emergence of diseases that cause significant economic losses and prevent the sustainable development of the aquaculture industry [1]. Thus, developing a safe and viable alternative of veterinary chemicals/antibiotics in aquaculture health management has received much attention [2]. The use of conventional chemotherapeutics including antibiotics and chemical disinfectants, not only pollutes the water, but also gradually causes the occurrence of resistant bacteria [3], and contributes to the accumulation of drug residues and reduces consumer preference for aquaculture products [4]. As a result, various alternative strategies for the antibiotic use have been proposed, among them, the use of pre- and pro-biotics having better efficacy. Probiotics are defined as live microbial feed supplements, and used as environment-friendly treatments to control diseases [5]. Previous research efforts have demonstrated that probiotics can improve disease resistance and immune responses of aquatic animals [6,7]. The most common probiotics used in aquaculture are *Lactobacillus spp.*, *Bacillus spp.*, *Vibrio spp.*, *Saccharomyces spp.* and *Enterococcus spp.* [8]. Moreover, the composition of an intestinal microbiota held by animals is often limited in several autochthonous species [9]. It has been suggested that the fish intestinal autochthonous bacteria might be a vital source of potential probiotics and some autochthonous bacteria might be treated as likely probiotics [10]. Studying the interaction between the gut autochthonous bacteria and the host immune system may reveal some beneficial or harmful effects exerted by these presumptive probiotics. Therefore, the investigation of the immunologic functions of autochthonous gut bacteria is relatively less [11]. The combined use of two or more probiotics and growth-promoting additives are new concepts in aquaculture [12]. Until now, experiments typically tested the effects of only one probiotic [13]. However, a few studies have combined different stimulants to amplify the impacts of cultured aquatic animals.

Evidence of the beneficial effects of probiotics gave birth to the concept of prebiotics [14], which are defined as indigestible (by the host) feed components that provide beneficial impacts to the host through their selective metabolism by favorable bacteria in the gastrointestinal tract (GI) [15]. β-glucans are prebiotics commonly used in fish and are naturally occurring polysaccharides found in the cell walls of the yeast *Saccharomyces cerevisiae*. Other sources, such as brewers' yeast, torula yeast *Candida utilis*, fungi, and algae, are also currently used [16]. A Previous study indicated that the use of prebiotics containing β-glucan and MOS was found to improve the immune system performance of common carp *Cyprinus carpio,* beluga *Huso huso*, and sea cucumbers *Apostichopus japonicus* [13,17,18].

Synbiotics, contain probiotics and prebiotics, have been introduced, and first used to enhance the immune responses of fish since 2005 [19]. In aquatic animals, it seems common to manipulate the gastrointestinal microbiota using synbiotics (probiotic with prebiotic) that alter the conditions of the gastrointestinal tract in favor of certain bacterial species. Mainly due to the synergistic effects of synbiotics, it may increase growth efficiency in endogenous populations, improve survival and increase nutritional supplementation for the living microbial population in the host gastrointestinal tract and reduce susceptibility to disease.

Although the use of single pre- and pro-biotics are now widely accepted in aquaculture, only a few studies have focused on the effects of synbiotics in farmed aquatic species, indicating that they might yield better results than the individual pre-and pro-biotics. This study was carried out to evaluate the effects of single or combined supplementation of two endogenous probiotics, along with the prebiotic (β-glucan). In this study we also aimed for the selection of a combination of prebiotic and probiotic to establish a suitable synbiotic formulation based on their effect on the intestinal flora and immunity in the rainbow trout. The candidate probiotics, two strains of *Lactobacillus plantarum* and *Lactobacillus pentosus,* were isolated from the gut of Tor grypus in our previous work [20,21].

Results

Bacteriological examinations and identification

Hematological indices were affected significantly in probiotic-treated experimental groups. Probiotic-fed groups showed a significant increase in RBC compared with the control group on day 60 ($p < 0.05$) (Table 1). The highest increase was observed in G5 and G8 groups, respectively.

Probiotic-fed groups had a significant difference in WBC count compared with the control group on day 60 ($p < 0.05$), and the highest WBC counts were observed in the G6 and G8 groups, respectively. The lowest WBC count was observed in G3 group after 60 days (Table 1).

Probiotic-fed groups had significantly different

levels of Hb than that in the control group on day 60 ($p < 0.05$), and the highest Hb levels were observed in G8, G4 and G7 groups on day 60, respectively (Table 1).

Nonspecific immunity parameters

Total Ig was significantly affected by diet in G6, and G7 compared to the other treatments ($p < 0.05$, Fig. 1). Moreover, a synergistic effect between *L. pentosus* and *L. plantarum* with β-glucan was observed. Anti-trypsin was higher in fish receiving dietary *L. pentoseus* with *L. plantarum* (G5) and *L. plantarum* in combination with β-glucan (G6), compared to fish fed the control diet and in other groups ($p > 0.05$, Fig. 2).

Among the non-specific humoral immune parameters, alternative complement activity was significantly boosted by a synergistic effect of β-glucan and the *L. pentosus* (G7) than dietary separately. Alternative complement activity was significantly higher in the serum of G 7 than that in the other treatments (Fig. 3).

The serum lysozyme activity increased marginally in all the treated groups at different times of trial feeding with some exceptions (Fig. 4). At day 60, G5 and G8 had higher serum lysozyme activity than other groups ($p > 0.05$).

After feeding the pro- and pre-biotic for 60 days, the ability to kill *L. garvieae* was more exceptional in the serum of G4, G5 and G8 than the control and other treatments ($p > 0.05$) (Fig. 5).

After sixty days of post-feeding (dpf), the G4, G5,

Table 1
Blood parameters in *O. mykiss* fed with different pro-, pre- and syn-biotic diets for 60 days.

Blood parameters	Groups	Time(0)	Time(60)
RBC ($10^6/\mu L^{-1}$)	Control	2.88 ± 0.38	3.43 ± 0.45^{ab}
	G2	3.15 ± 0.32	2.31 ± 0.89^{b}
	G3	3.68 ± 0.36	3.23 ± 1.46^{ab}
	G4	3.35 ± 0.32	2.89 ± 0.94^{ab}
	G5	3.17 ± 0.36	3.74 ± 0.83^{a}
	G6	3.77 ± 0.45	2.46 ± 1.16^{ab}
	G7	3.44 ± 0.27	3.04 ± 0.58^{ab}
	G8	2.97 ± 0.36	3.66 ± 0.55^{a}
WBC ($10^3/\mu L^{-1}$)	Control	13.33 ± 1.61	13.50 ± 1.81^{b}
	G2	12.2 ± 3.20	15.25 ± 2.62^{ab}
	G3	11.33 ± 1.61	13.35 ± 8.81^{b}
	G4	15.2 ± 3.20	19 ± 5.17^{ab}
	G5	12.625 ± 3.95	15.9 ± 6.82^{ab}
	G6	15.1 ± 3.53	21 ± 5.11^{a}
	G7	17.1 ± 2.03	19.5 ± 6.51^{ab}
	G8	12.25 ± 3.75	22.5 ± 7.5^{a}
Hb (g/dl)	Control	9.11 ± 1.09	11.63 ± 0.98^{ab}
	G2	10.51 ± 1.09	11.64 ± 0.36^{ab}
	G3	11.65 ± 1.13	10.90 ± 0.81^{b}
	G4	11.07 ± 1.01	12.46 ± 1.05^{a}
	G5	10.37 ± 1.01	11.44 ± 1.04^{ab}
	G6	11.55 ± 1.36	11.42 ± 0.53^{ab}
	G7	11.21 ± 1.09	12.22 ± 0.46^{ab}
	G8	9.22 ± 1.02	12.48 ± 0.65^{a}

*Each value represent as a mean ± standard error (n = 9). Different lowercase superscripts denote significant differences within columns ($P < 0.05$).

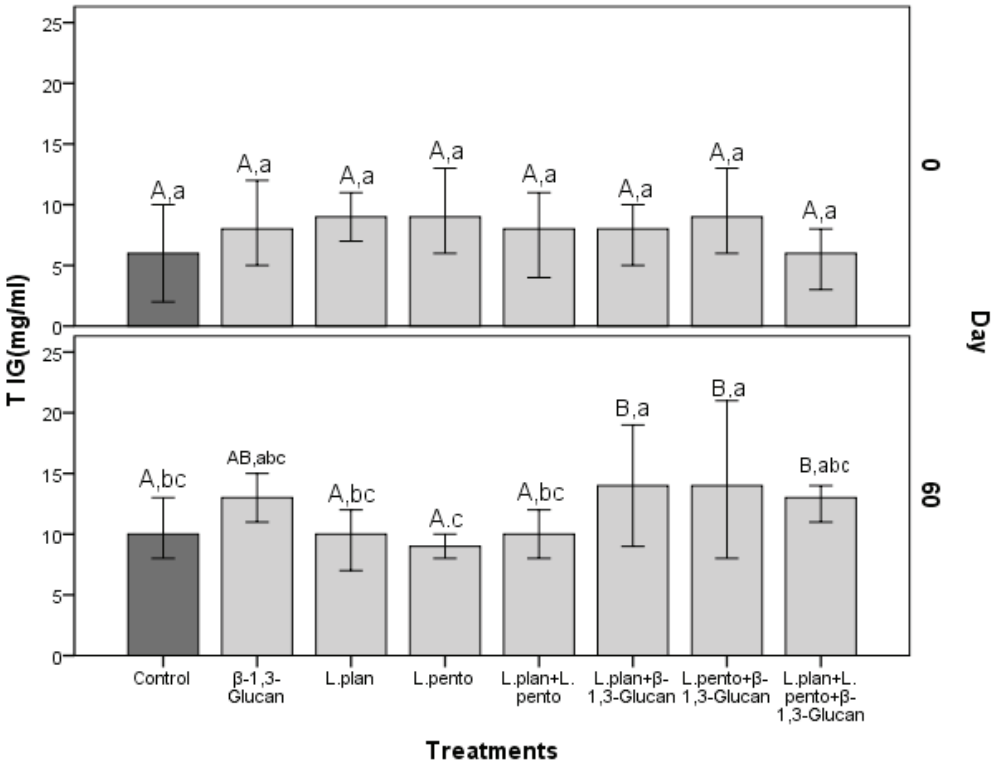

Figure 1
Total Ig of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).

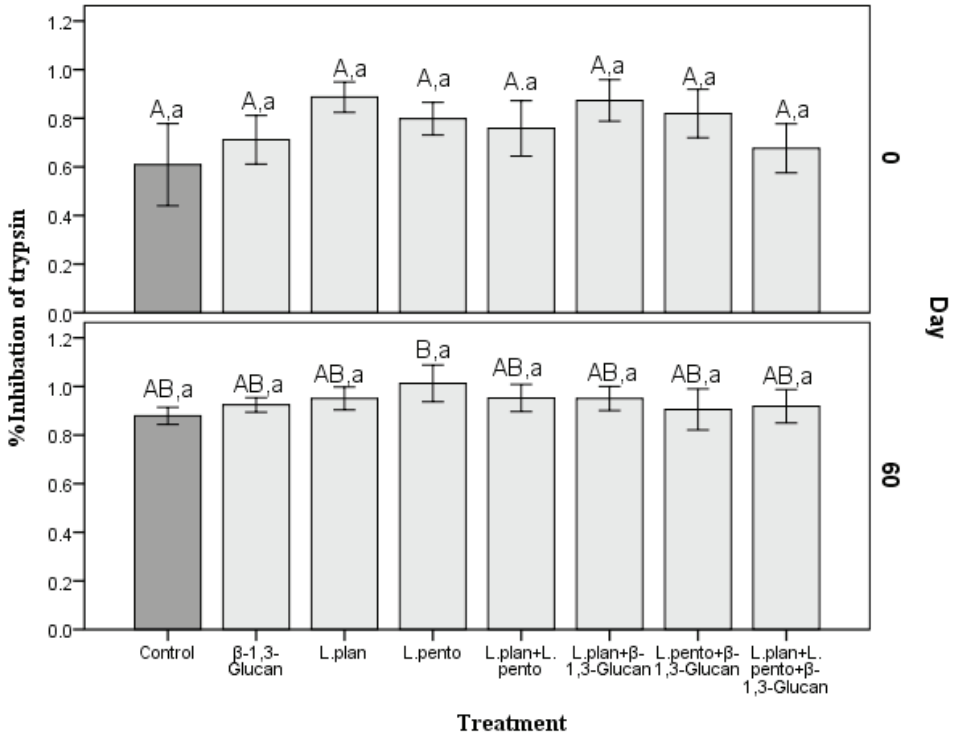

Figure 2
Anti-trypsin activity of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).

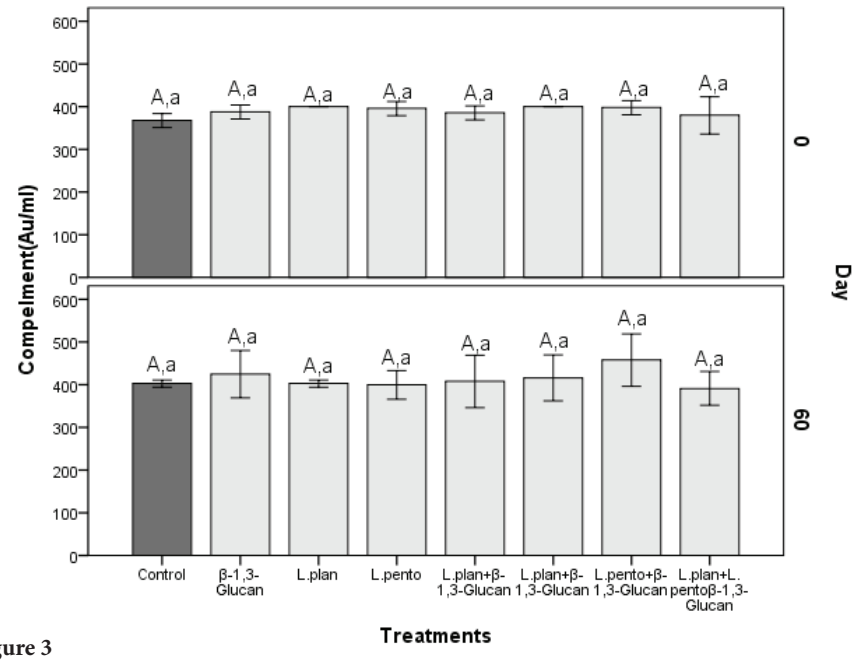

Figure 3
Serum complement activity of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).

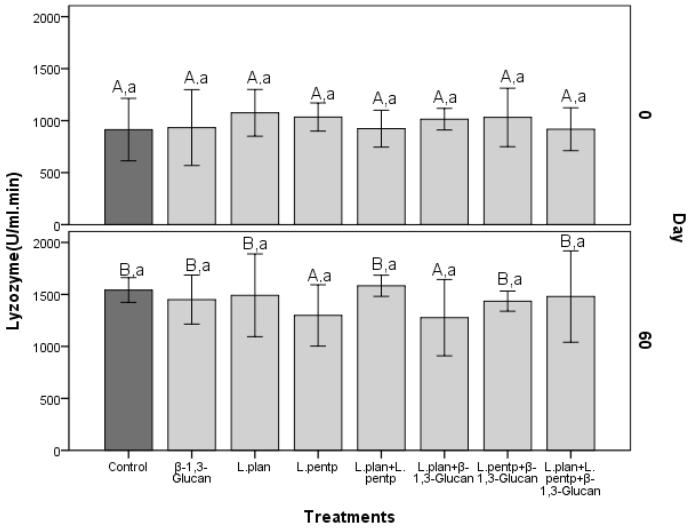

Figure 4
Serum lysozyme activity (unit/ml^{-1}) of O. *mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).

and G8 showed higher oxide anion production upon stimulation with pro- and pre-biotics compared with the control group (Fig. 6).

Serum myeloperoxidase content was positively affected by both *L. pentosus* alone and *L. pentosus* with *L. plantarum* and β-glucan diets (Fig. 7). It was higher in fish fed in G3, G5 and G7 groups than those in the control group ($p > 0.05$).

Serum Anti-trypsin content was positively affected by both *L. plantarum* alone and *L. pentoseus* with *L. plantarum* and *L. plantarum* with β-glucan diets (Fig. 8). It was higher in fish fed with G3, G5, and G6 diets than fish fed with the control diet ($p > 0.05$).

Microbiological assay

Before the pro- and pre-biotic feeding, the fish, showed a low detectable LAB level in the entire intestines. Although in G1 an increase in viable counts at day 60 was seen, the only viable count of LABs significantly increased in a time-dependent manner in the intestine of G8 (Table 2). Content of the intestinal bacteria counts in fish fed with pro- and pre-biotics showed significant differences compared with the control ($p < 0.05$).

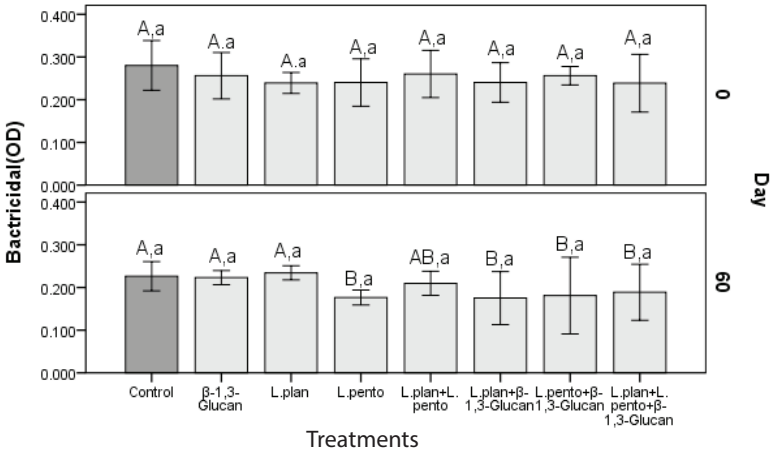

Figure 5
Serum bactericidal activity of *O. mykiss* in eight experimental groups . Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).

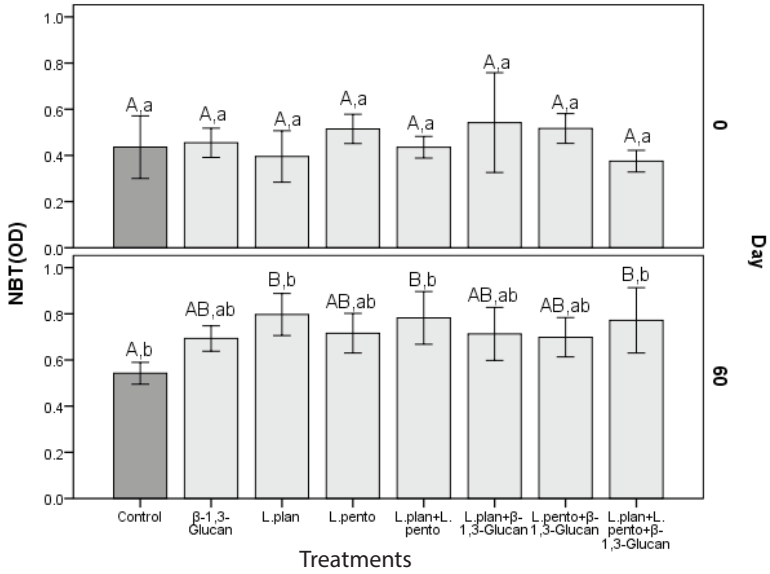

Figure 6
Reduction in NBT of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).

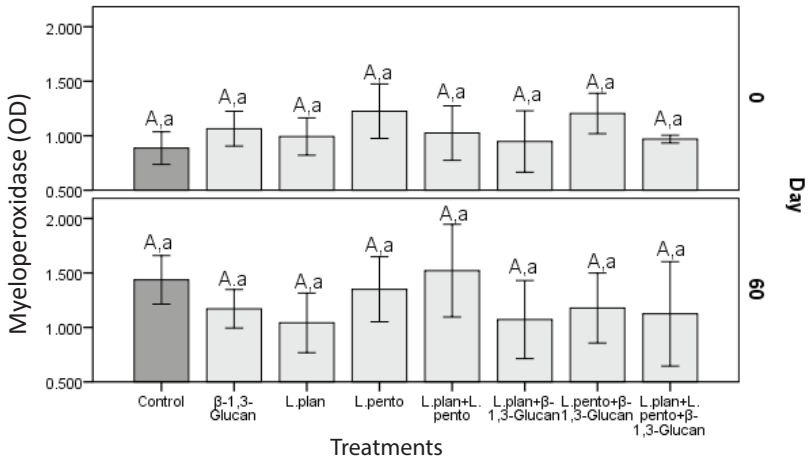

Figure 7
Serum myeloperoxidase activity of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).

Discussion

This study was performed to assess the effects of two indigenous bacteria *L. plantarum* and *L. pentosus* separately and combined with β-1,3-glucan (a yeast cell wall-derived immune stimulant) on the general innate immune system and microbial flora in rainbow trout (*O. mykiss*). The results demonstrated that continuous feeding of symbiotic (pre- and pro-biotic) for 60 days led to increased several immune accelerated parameters of rainbow trout.

β-1,3-glucan alone cannot be hydrolysed by pancreatic or brush border digestive enzymes in the proximal intestinal tract of human and fish. β-1,3-glucan has repeatedly been shown to stimulate innate im-

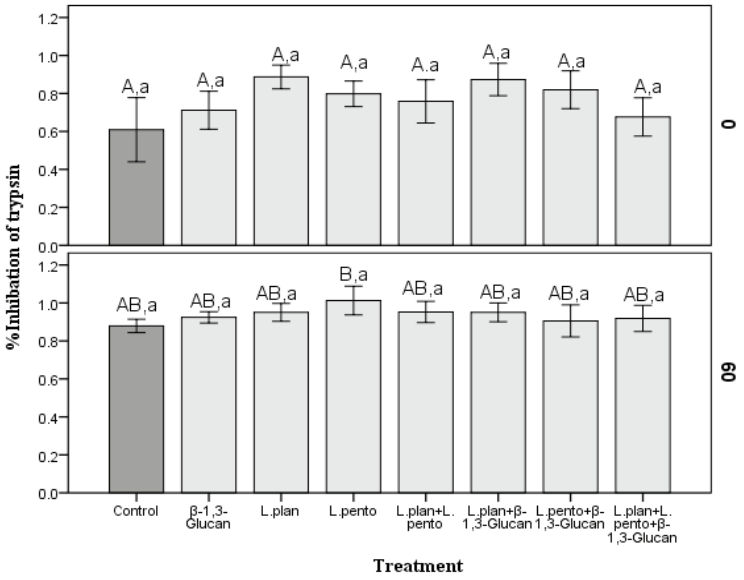

Figure 8
Anti-trypsin activity of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments (p <0.05).

Table 2
Total viable counts and total lactic acid bacteria (LAB) from the digestive tract of *Tor grypus*. MRS: de Man, Rogosa and Sharpe. Values are presented as mean ± SD (n = 9). Different small alphabetic letters in the same column show significant differences (p < 0.05).

Groups	Groups	Time (0)	Time (60)
Total counts 10^5 cfu mL^{-1}	Control	34.33 ± 2.08	27 ± 8.50^{ab}
	G2	28.2 ± 6.53	12.83 ± 3.31^{c}
	G3	23.25 ± 7.13	29.16 ± 5.81^{ab}
	G4	26.2 ± 6.53	38.5 ± 7.14^{a}
	G5	28.2 ± 6.53	24.33 ± 6.88^{bc}
	G6	23.25 ± 7.13	26.83 ± 9.74^{ab}
	G7	26.2 ± 6.53	21.83 ± 5.45^{bc}
	G8	27.66 ± 7.76	19.33 ± 4.36^{bc}
MRS counts (Lactobacillus) 10^2cfu mL^{-1}	Control	1/2 ± 0/44	1/2 ± 0/54^{e}
	G2	1/5 ± 0/7	5/2 ± 1/48de
	G3	1/33 ± 0/57	23/66 ± 5/08^{b}
	G4	1 ± 0	9/83 ± 2/48^{cd}
	G5	1/25 ± 0.5	12/16 ± 4/66^{cd}
	G6	1 ± 0	7/5 ± 2/88^{cde}
	G7	1/25 ± 0/5	13 ± 1/54^{c}
	G8	1 ± 0	33/66 ± 6/08^{a}

mune functions in various fish species [22, 23], and the expected activities were confirmed in *O. mykiss*. In this study, immune parameters examined, including lysozyme, and alternative complement pathway (ACP) activity, respiratory burst, bactericidal activity, and total Ig in serum, were stimulated by β-1,3glucan more than the control group. Overall, these results confirm that β-1,3-glucan can be an efficient stimulant of innate immunity in *O. mykiss*, as demonstrated previously in other studies [24]. Improvement of the immune system in fish fed with a β-1,3-glucan diet might be attributed to fermentation in the large intestine or colon by lactic acid-producing bacteria (LAB), enhancing their relative populations, elevated health status and increased colonization of the LAB compared to the control diets.

Regarding hematological parameters of rainbow trout in experimental groups, the *Lactobacillus* probiotics (*L. plantarum* and *L. pentusus*) not only act as immune promoter, but also cause higher RBC, WBC, hematocrit and hemoglobin (Table 1). Blood parameters are essential tools for assessment of the physiological stress response and general health conditions of fish during nutritional and environmental changes [25]. In the current study, G8 treatment significantly affected RBC of rainbow trout. Similarly, Rodriguez-Estrada et al. [26] reported that hematological indices were enhanced by the supplementation of inactivated *Enterococcus faecalis* in rainbow trout diets. The higher RBC count in the blood of fish fed with diets supplemented with 1×10^8 CFU/g^{-1} of *Lactobacillus* indicated stimulation of fish defensive mechanisms against pathogens, positive effect on fish health and the improvement of the immune functions of blood [27, 28]. The increase in the number of WBC in fish fed with probiotics may indicate stimulation of the innate immune system, possibly resulting in the improvement of the defensive systems against environmental stress or pathogens, because WBCs are regarded as the first lines of defense [29]. Similarly, significant enhancements of total leukocytes number in *Oreochromis niloticus* [30] and *Oncorhynchus mykiss* [31] fed with probiotic supplemented diets have been reported. According to Silva et al. [32] and Falcon et al. [33], the reduction in the total number of circulating cells implies downfall of immune resistance predisposing animals to the pathogenic infection. This indicates that the fish that were fed the β-1,3-glucan diet (G2) and 1×10^8 CFU/g^{-1} of *L. plantarum* (G3) are more susceptible to possible disease outbreaks than those fed with the other doses (Table 1). Moreover, the reduction in hematocrit percentage in control group (Table 1) may indicate that they are more vulnerable to stress arising from the experimental management or the pathogenic load naturally present in the culture environment [34]. This is also indicated by other blood variables analyzed in this study. The hemoglobin content in the blood plays a vital role and serves as oxygen transport element to the body tissues. However, it should be noted that the *L. plantarum*, in combination with β-glucan, had the highest levels of hemoglobin. The increase in its contents indicates a greater supply of oxygen to the fish and consequently, improves the welfare of fish [27]. This demonstrates that *Lactobacillus* supplementation increases the availability of oxygen in fish blood, resulting in beneficial health effects.

The results of the present study showed that fish fed diets supplemented by *L. plantarum* and *L. pentoseus* along with β-glucan showed higher serum lysozyme activity than the other groups ($p < 0.05$). Accordingly, the more elevated WBC measured was concomitant of increased serum lysozyme activity in all treatments. The use of probiotic and synbiotic has been carried out in Nile tilapia (*Oreochromis niloticus*) [35,36,37], in rainbow trout (*Oncorhynchus mykiss*) [38], rockfish (*Sebastes schlegeli*) [39], in Atlantic salmon (*Salmo salar*) [40], in the yellow croaker *Larimichthys crocea* [41], and in the Japanese flounder *P. olivaceus* [42] *Epinephelus coioides* [43], and *Cyprinus carpio* [44].

The presence of protective proteins in fish blood such as complements, acute phase proteins, lysozyme, transferrin and anti-proteases can be evaluated by serum bactericidal activity, which are considered nonspecific responses to inhibit the growth of infectious microorganisms [45]. Results of the current study revealed that serum bactericidal activity elevated in fish which were fed with probiotic (G4 and G5) and synbiotic enriched diets (G8) compared with the control group and G2 (β-1,3-glucan) similar to previous reports in other fish species fed with probiotics [35,46]. Improvement of the immune system in fish fed a synbiotic diet and indigenous probiotic, which might be attributed to elevated health status, increased colonization of the probiotic compared with the control diets. Moreover, fish fed synbiotic diet had the highest serum bactericidal activity, which was associated with the highest serum lysozyme and complement activity in these groups. It has been suggested that synbiotics can induce immune system by short-chain fatty acids, which can partially act as a source of energy for intestinal epithelial cells and also may have a role as a messenger between gut microbiota and immune system by modulation of signaling and transcriptional pathways [47]. In the absence of specific opsonization, alternative immune responses could depend on the presence of mannose receptors and toll-like receptors (TLRs) in microbes, which bind to mannose and glucans, leading to enhanced phagocytic and bactericidal abilities in phagocytes and neutrophils (48,49). Mannan oligosaccharides bind with and block receptors on pathogens, preventing their colonization or invasion of the host. MOS also enhances the liver's secretion of material rich in mannose-binding lectin, which binds the bacterial capsule and triggers the complement cascade [50].

The alternative complement pathway activity (ACP) can be measured through the determination of serum hemolytic activity in response to foreign red blood cells [23]. We found that fish fed diet supplemented with β-1,3-glucan and *L. pentosus* (G7) had higher serum hemolytic activity than fish in control and the group fed with *Lactobacilus plantarum* + *Lactobasilus pentoseus* with 1% β-glocun (G8). Similarly, Van Doan et al. [36] reported that inclusion of 10 g/kg^{-1} LMWSA in the diet significantly improved serum ACP in Nile tilapia compared with fish fed 20 and 30

Table 3
primers sequences that were used for identification of different lactoacid bacteria

primer	5'Sequence3'	Specificity
forward	GCCGCCTAAGGTGGGACAGAT	*L. plantarum*
Revers	TTACCTAACGGTAAATGCGA	
	CGCCGCCCGGGTGAAGGTG	
Forward	CTGCTGGGACGAAAAG	*Lb pentoseus*
Revers	CTGCTGGGACCTTAA	

g/kg^{-1} LMWSA. These results were also in accordance with the previous results in different species fed diet supplemented with sodium alginate [41,51]. On the other hand, the finding of the current study showed that fish fed the diet supplemented with *L. pentosus* had the lowest serum haemolytic activity in comparison with other experimental groups as also in green terror (*Aequidens rivulatus*) [52]. In line with the results of this study, it has been reported that PA in combination with GOS significantly increased ACP in the rainbow trout [53] and rockfish [39] compared with those in the control pro- and pre- fed fish.

Supplementing diet with synbiotics (β-1,3-glucan /*L. pentosus* and β-1,3-glucan *L. plantarum*) in the rainbow trout affected serum globulin level. Present results concur with the reports of previous researchers that using immunostimulants increased total serum protein, albumin, globulin, and immunoglobulins in different fish species [54,55,56].

This study demonstrated that the combined use of β-1,3-glucan with *L. pentosus* and *L. plantarum*, positively affected cellular immunity as well as humoral immunity of juvenile rainbow trout. The effect measured in the synbiotic-fed group with the exception of NBT, anti-trypsin and bactericidal activity, was higher than that predicted by the individual effects measured in the prebiotic and probiotic-fed group. This study represents the synergistic effects of *Lactobacillus plantarum* and *Lactobacillus pentoseus* with β-1,3-glucan.

The intestinal microbiota of fish plays a key role in nutritional function, enhances growth performance, and stimulates the host immune system and resistance against pathogens [53]. Singular or combined administration of β-1,3-glucan and probiotic *L. pentosus* and *L. plantarum* were showed an interesting and remarkable result in the number of lactic acid bacteria in the rainbow trout intestine. The *Lactobacillus plantarum* + *Lactobacillus pentosus* with 1% β-glucan (G8) had a significantly increased LAB in the experimental groups. But the interesting point was that the *Lactobacillus plantarum* group (G3), when used alone, had a better performance than combined administration of β-1,3-glucan (G6) in the number of LAB. In conclusion, the ability of *Lactobacillus plantarum* to colonize and modify the intestinal microbiota as a potential probiotic strain, was confirmed. Dietary fermentable β-1,3-glucan are components that are not hydrolyzed by digestive enzymes of non-ruminant animals such as fish and consequently are the main substrates for bacterial fermentation in the gut [57]. Therefore, an increase in gut microflora of G8 may have occured [58]. In our study, the higher degree of adhesion of specific microbes that are supplemented through diets may be the reason for enhanced innate immune responses and hematological indices of fish.

In conclusion, the diet administration of *L. plantarum* and *L. pentosus* that showed to colonize and modify the intestinal microbiota as a potential and host derived probiotic strain, was confirmed. The selected probiotic strains isolated from fish are safe and capable of surviving and colonizing the fish intestinal mucus, as well as antagonizing the resident microbiota. The results of the present study strongly suggest that the dietary combination of *L. plantarum*, *L. pentosus* and β-1,3-glucan is significantly effective to stimulate some hematological factors of *O. mykiss*, compared with their singular administration. The *Lactobacillus plantarum* + *Lactobacillus pentosus* with 1% β-1,3-glucan (G8) caused significant alterations in the intestinal microbiota by significantly decreasing the bacterial diversity, demonstrated by reducing the total number of bacteria than the control group and boosted immune responses of rainbow trout. Therefore, they could be considered as a useful alternative to chemotherapeutic treatments to promote fish health status. Future researches are needed to be carried out under a more holistic approach, in which different rearing conditions (i.e., stocking densities, water temperatures, oxygen levels and exposure to pathogens) and concomitant use of antibiotics or vaccines and indigenous probiotics are used. In addition, the study of the colonization of these bacteria in the digestive

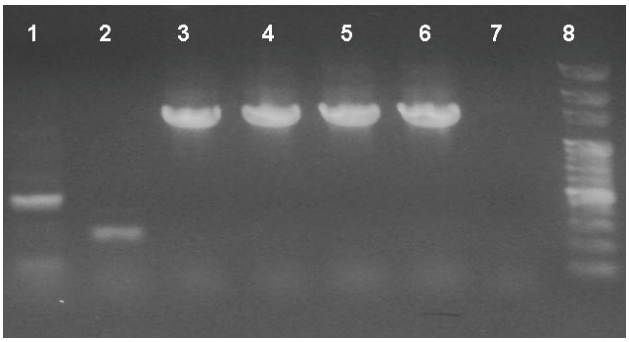

Figure 9
PCR amplification of 16 SrRNA genes for the identification of lactobacilli species. 1: PCR products of isolate 1 suspected of Lactobacillus pentosus with species-specific primers (465 bp), 2: PCR products of isolate 2 suspected of Lactobacillus plantarum with species-specific primers (284 bp), 3, 4, 5 and 6: PCR products on DNA isolated from suspected bacteria with universal lactobacilli primers, 7: negative control, 8: M: molecular marker 100 bp.

system by the method of DGGE is needed in order to provide a more comprehensive analysis on the effects of combined use of these probiotics and the prebiotics.

Material and methods

Isolated bacteria

Lactobacillus plantarum subsp. *Plantarum* and *Lactobacillus pentoseus* were used in food supplementation. Bacterial identification was primarily performed based on colony and cell morphology, Gram staining, and biochemical testing and according to their high in vitro probiotic characteristics. The confirmation of the probiotic bacteria isolated from the intestine of *Tor grypus* was performed using PCR analysis for the ribosomal RNA (rRNA) gene, as described by Mohammadian et al. [46]. The sequences of primers used in this study are shown in Table 3. The PCR was carried out on a PC 707 thermal cycler (Termocycler, Mastercycler Gradient, Eppendorf, Germany). PCR reaction was performed in a final volume of 25 µL containing 3 µL cDNA and 22 µL PCR mixture consisting of Taq DNA polymerase (1 unit), forward and reverse primers (100 nM), 1X PCR buffer, $MgCl_2$ (2 mM), and dNTPs (100 mM). The PCR was performed after 2 min of initial denaturation at 92°C, and 35 cycles of 30 s of denaturation at 95°C, 45 s of the annealing at 57°C, 45 s of primer extension at 72°C and 5 min of final extension. Amplification products were analyzed by electrophoresis in 1% (w/v) agarose gel (Fig. 9) containing ethidium bromide (1 mg/ml^{-1}) [23].

Experimental Setup

The experiment was conducted at the laboratory of the aquatic health of The Veterinary Faculty of Chamran University of Ahwaz, Iran. Six hundred juvenile rainbow trouts (10.4±2.4 g) were randomly divided into eight experimental groups with three replicates each following a complete block design.

Experimental diet of each group were as follows: basal diet (G1: control), basal diet supplemented with 1% β-1,3-glucan (G2), *Lactobacillus plantarum* (G3), *Lactobacillus pentoseus* (G4), *Lactobacillus plantarum* + *Lactobacillus pentoseus* (G5), *Lactobasilus plantarum* with β-1,3-glucan (G6), *Lactobacillus pentoseus* with 1% β-1,3-glucan (G7) and *Lactobacilus plantarum+Lactobasilus pentoseus* with 1% β-1,3-glucan (G8).

Rearing

Fish were obtained from local fish farming and the healthy fish were kept and acclimatized in 1000-L water tanks for 2 weeks. Fish health status was verified by physical examination (excess of mucous secretion, normal coloration, erosion of scales or fins, skin, bulging of eyes and presence of cysts, spots or patches over the body and gills) and behavioral signs (swimming and feeding reflexes). Water quality parameters including dissolved oxygen (DO) and pH were measured daily, whereas nitrate, nitrite and ammonia concentrations were monitored bi-weekly. Nitrate, nitrite and ammonia concentrations were determined using a portable Hach DR/2400 spectrophotometer. Water temperature, DO, and pH ranged from 14.8-19.4 °C, 6.6-6.9 mg/L^{-1}, 6.84-7.06, respectively.

Microorganisms

The potential probiotic bacteria *Lactobacillus plantarum* and *L. pentaseus* were previously isolated from the gut contents of healthy *Tor grypus* and identified as potential probiotics based on diverse *in vitro* tests [23]. De Man, Rogosa and Sharpe broth (MRS) were used to grow *L. plantarum* and *L. pentasus* strains (48 h at 25 ˚C). Bacterial density was estimated via McFarland standard tube No. 0.5, OD= 0.132 at 600 nm and correlated with colony forming unit (CFU) counts using serial dilution and spread plating on MRS agar. The bacteria were subsequently harvested by centrifugation at 1500 g for 15 min in sterile phosphate-buffered saline (PBS). All prepared diets were packed in sterile propylene containers and stored at 4°C for weekly use.

Sample Collection

Blood samples were collected at day 0 and 60 of the experimental period. At the end of the trial, five apparently healthy fish (no obvious skin lesions) from each replicate tank (15 fish per treatment) were anesthetized with 400 mg/L^{-1} phenoxy ethanol. The blood samples were withdrawn from the caudal vein using 1.0 ml non-heparinized syringes. A part of collected blood was transferred into heparinized microtube and kept on ice for further hematological assay and the residue was allowed to clot at room temperature (for 60 min) and subjected to centrifugation (3000 g, 10 min, 4°C) to separate serum. Serum samples were stored at 80°C for further analysis.

Hematological Parameters

The blood was diluted with appropriate diluting fluids and RBC and WBC counts were determined using improved Neubauer hemocytometer [59]. Hemoglobin concentration (Hb) was measured spectrophotometrically (Jenway 6400, UK) at 540 nm by the cyanomethemoglobin method [60]. Hematocrit percentage (Hct%) was measured with the microcentrifuge method (Micro-hematocrit centrifuge, 346, UNIPAA, Poland) for 10 min in duplicate.

Immunological Parameters

The effect of treatments on immune responses was evaluated by assessment of the lytic activity of lysozyme against *Micrococcus lysodeikticus* [61] and oxidation of the tetramethyl-benzidine by myeloperoxidase enzyme [62]. Nitrobluetetrazolium (NBT) reduction assay was carried out using the spectrophotometric method [46]. The lysis of the 1% rabbit red blood cells and 1% sheep red blood cells coated with rabbit anti-sheep erythrocyte based on Leiro et al. [63] were detected as the activity of the alternative and classical pathway of complement, respectively. The active and heat-inactivated serum bactericidal effects against *L. garvieae* were evaluated using the broth-microdilution method [64]; the differ-

ence between the activity of the active and inactivated serum was accounted as the antibacterial activity of each sample. The zinc sulfate precipitation method [65] was used for the measurement of the total immunoglobulin.

The humoral immune response against *L. garvieae* was detected using the micro-agglutination test [66]. Serum anti-protease activity was performed [66] by incubating 10 µl of serum with 20 µg of trypsin dissolved in 100 µl of Tris-HCl (50 mM, pH 8.2). In blank serum, 100 µl of Tris-HCl was added to 10 µl of serum, instead of trypsin in Tris-HCl, and in the positive control, no serum was added to trypsin. All tubes were made up to 200 µl with Tris-HCl and incubated for 1 hour at room temperature. After the incubation, 2 ml of 0.1 mM substrate BAPNA (Na-benzoyl-DL-arginine-p-nitroanilide HCl, Sigma chemicals), dissolved in Tris-HCl (containing 20 mM calcium chloride), was added to all tubes and incubated for further 15 minutes. At the end of incubation, the reaction was stopped by adding 500 µl of 30% acetic acid. The optical density was measured at 410 nm by using a UV-Visible spectrophotometer (Shimadzu UV-1601). The percentage trypsin inhibition was calculated from the following formula: Trypsin inhibition (%) = (A1-A2/A1) x 100, where A1 = control trypsin activity (without serum); A2 = activity of trypsin remained after addition of serum.

Bacterial Community Analysis

Micro floral analysis was done as described by Mohammadian et al. [67]. Total and *Lactobacillus* counts in fish intestines were determined by plate counting on TSA and MRS agar, respectively. The intestine of the experimental fish (6 fish from each treatment) was sampled just prior to starting on the experimental diets, and 60 days past the probiotic and prebiotic feeding and to determine the effect of both combination (symbiotic effect), the supplemented diets were stopped for 24 h [67,68]. This was done by aseptically dissecting the fish after overdose (1 ml/L^{-1}) of anesthesia (Benzocaine; Sigma-Aldrich Co., St Louis, MO, USA) and removing a portion of posterior intestine that was finely chopped. All steps were carried out under sterile conditions. One gram of the sample was homogenized with 9 ml of sterilized phosphate buffered saline (PBS, 0.1 M, pH=7.0) and stirred for 1 min in stomacher (Orugan Stomacher, Tokyo, Japan). Subsequently, dilution series were prepared from the homogenate and plated in the MRS and TSA media. The plates were incubated at 30°C for 48 h before counting. Confirmation of the isolated bacteria from the gut was done in the previous work by using morphological, biochemical, and molecular tests adopted from Bergey's manual of systematic bacteriology [67,69].

Statistical analysis

All statistical tests were performed using SPSS software (SPSS, Release 16.0, SPSS, Chicago, IL, USA). Two-way analysis of variance (ANOVA) and general linear model was used to evaluate the effect of time and treatments on each variable. A one-way analysis of ANOVA was done to determine the differences between different variables. Differences were considered statistically significant when p <0.05, and the results were expressed as mean ± SD.

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Author Contributions

T.M. and M.A. designed and supervised the studyand wrote and revised the manuscript draft. M.M. conceptualized the study. M.kh. conducted *in vitro* evaluations of probiotic candidates.

Conflict of Interest

The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

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Effect of *Aloe vera* and *Salvia officinalis* extract supplemented diet on hematology, histopathology, and hypoxia resistance in rainbow trout

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ABSTRACT

This study investigated the effects of *Aloe vera* and *Salvia officinalis* extracts on hematological and haemato-biochemical parameters, histopathology, and resistance against hypoxia stress in *Oncorhynchus mykiss*. The rainbow trout (10 ± 0.1 g) were fed seven different diets supplemented with 0% (control), 0.5%, 1% and 1.5% of *A. vera* or *S. officinalis* extract for 30 days. The feeding in all treatments was continued for 2 weeks using control diet. The fish blood samples were collected on days 30th and 45th, and total red blood cells, hematocrit and hemoglobin were measured. The glucose and cortisol of serum were examined just before and one hour after hypoxia stress (3 mg L^{-1}) at days 30th and 45th. The gill tissue samples were taken from all treatments for histopathological study at the end of experimental period. The *S. officinalis* (0.5%) treated group showed a significant increase ($p < 0.05$) in red blood cells, hematocrits and hemoglobin compared to control group. While the *A. vera* (1 and 1.5%) treatments revealed significant increase in hematocrit and hemoglobin concentration compared to the control group ($p < 0.05$). Moreover the glucose and cortisol levels of serum were increased significantly only in *S. officinalis* (0.5%) treated group after hypoxia stress on days 30th and 45th compared to the control group ($p < 0.05$). No serious histopathological changes were observed in any treatments and control group. Based on the results obtained, dietary *S. officinalis* (0.5%) hydroethanolic extract improved the haematological and haemato-biochemical parameters and increased the rainbow trout resistance against hypoxia stress.

Keywords

Oncorhynchus mykiss, Herbal Extracts, Environmental Stresses, Hematology, Histopathology

Abbreviations

RBC: red blood cell
S. officinalis: Salvia officinalis
O. mykiss: Oncorhynchus mykiss
A. vera: Aloe vera

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Introduction

Rainbow trout (Oncorhynchus mykiss) is a very popular coldwater species in world aquaculture industry. Sustainable development in rainbow trout culture depends on the special attention on health status of this fish [1]. Therefore, a new approach to immunotherapy is actively used to treat or protect fish when exposed to stresses. In this regard, extensive research has been carried out to test various immunostimulants including medicinal plants which they have found to be effective in fish [2]. It has been found that medicinal herbs enhance the immune system against stressors and especially various bacteria[3, 4]). Modulation of the immune response using medicinal plant products as a therapeutic measure has become the focus of extensive scientific investigation [5]. Two common themes running through recent research is the immunomodulatory properties of Aloe vera [6] and Sage, Salvia officinalis [7].

A. vera (synonym: Aloe barbadensis Miller) belonging to the family Liliaceae is widely distributed in the tropical and subtropical regions of the world, and the genus Aloe contains over 400 different species and Aloe barbadensis Miller is considered to be the most biologically active [8]. S. officinalis L. (sage, garden sage, or common sage) from the family Lamiaceae, is a worldwide cultivated aromatic herb. It is native to the Mediterranean region and is currently cultivated in various countries [9]. These plants, with their medicinal potency, originated from their bioactive content, play an important role in preventing diseases and enhancing the ability of the aquatic immune system [10]. Many researchers have pointed to the importance and role of these plants in enhancing growth indices [11] and immune responses [12] and modifying the histopathological lesions of some fish species [13].

Although researchers have reported in many cases the role of medicinal plants (such as A. vera and S. officinalis), and despite of the effect of their natural bioactive content in development of fish prevention and safety, they should not be ignored by their potential adverse effects. Therefore, besides the use of medicinal plants in aquatics, it is important to study their effect on the vital tissues of fish [14].

Histopathological changes have been widely used as biomarkers in the evaluation of the health of fish exposed to contaminants, chemical and natural materials both in the laboratory [14] and field studies [15]. The gills, which participate in many important functions in fish, such as respiration, osmoregulation, and excretion, remain in close contact with the external environment, are particularly sensitive to changes in the quality of water, and are considered the primary target of the contaminants. So, in herbal extracts studies, the histopathological assays would be useful to evaluate the effect of plant extracts on gill tissue.

The objective of the present study was to evaluate the effects of A. vera and S. officinalis ethanolic extract on various haematological and histopathological parameters and stress indices in O. mykiss to develop an alternative drug to enhance fish resistance against hypoxia in aquaculture.

Results

The chemical composition of herbal extracts was determined by GC/MS and presented in Table 1. The main components were n-Hexadecanoic acid (15.22%), Cineol (10.35%), Oleic acid (12.28%) followed by Octacosane (10.71%) for *A. vera*; and 1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (E)- (15.51%), á-Sesquiphellandrene (12.26%), Cineol (12.21) followed by 1-Naphthaleneacetic acid, methyl ester (10.10%) for *S. officinalis*. Cineol is a monoterpenoid and exists in both *A. vera* and *S. officinalis* extracts in this study (Table 1).

Data of Hematological parameters of trial and control groups are compared statistically in Table 2. According to the results of Table 2, the fish treated with *S. officinalis* (0.5%) revealed the highest value of total RBC, hematocrit, and hemoglobin in both 30 and 45 sampling days, showing a significant difference compared to the control group. Moreover at day 45 the fish fed with diets supplemented with, *S. officinalis* (0.5%) exhibited significantly higher hematocrit and hemoglobin values compared to the control group. At day 30 *A. vera* (1 and 1.5%) treatments were significantly different in comparison to the control group just in terms of hematocrit percentage. Also, *A. vera* (1%) and *A. vera* (0.5 and 1%) treatments were significantly different with the control group in cases of hematocrit and hemoglobin values, respectively (Table 2).

Glucose and cortisol levels of serum samples of fish in all groups were measured on days 30 and 45, prior and one hour after exposure to hypoxia stress (3 mg/L^{-1}), and the results are shown in Table 3. There was no significant differences between the glucose and cortisol levels of fish in all treatments and control groups before stress at day 30th and 45th. Also, no treatment showed any difference with the control group in case of glucose and cortisol levels after stress at day 45. The *S. officinalis* (0.5%) treatment showed a significant difference in serum glucose and cortisol levels after exposure to hypoxia stress on day 30 compared to the control (Table 3).

Cumulative mortality of fish at the end of 6 hours of hypoxia stress on days 30 and 45 are illustrated and compared in Figure 1 and 2, respectively. The *S. officinalis* (0.5%) treatment showed significantly lower

Table 1
The main chemical composition of *A. vera* and *S. officinalis* extracts

S. officinalis		*A. vera*	
Component	**%**	**Component**	**%**
Cineol	12.21	p-Xylene	2.25
Bicyclo[3.1.0]hexan-3-one, 4-methyl-1-(1-methyleth-yl)-, [1S-(1à,4á,5à)]-	2.14	1,5-Heptadien-4-one, 3,3,6-trimethyl-	2.41
Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (ñ)-	4.71	Oleic acid	12.28
Borneol	5.29	1-Heptanol,2-propyl-	2.32
Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-meth-ylene-	4.75	Tetradecanoic acid	2.28
1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (E)-	15.51	n-Hexadecanoic acid	15.22
Germacrene D	2.22	Squalene	5.37
á-Sesquiphellandrene	12.26	Hentriacontane	5.99
1-Naphthalenol, 4-methoxy-	9.55	Octacosane	10.71
1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-te-tramethyl-, [1ar-(1aà,4á,4aá,7à,7aá,7bà)]-	5.38	1,2-Benzenedicarboxylic acid, diisooctyl ester	9.50
Butanoic acid, 3-methyl-, 1-ethenyl-1,5-dimeth-yl-4-hexenyl ester	3.27	Eicosane	4.87
1-Naphthaleneacetic acid, methyl ester	10.10	Heptacosane	5.19
1-Naphthalenepropanol, à-ethenyldeca-hydro-à,5,5,8a-tetramethyl-2-methylene-, [1S-[1à(R*),4aá,8aà]]-	3.39	Acemannan	5.97
Squalene	2.22	Cineol	10.35

Table 2
Hematological parameters of different groups in day 30 and 45

Treatments	**Blood Index**					
	Total RBC (10^6 Cell/µl)		**Hematocrit (%)**		**Hemoglobin (g/dl^{-1})**	
	Day 30	**Day 45**	**Day 30**	**Day 45**	**Day 30**	**Day 45**
Control	1.28 ± 0.13^{a}	1.33 ± 0.13^{a}	41.22 ± 1.09^{a}	41.11 ± 0.78^{a}	7.77± 0.68^{a}	6.78 ± 0.52^{a}
S. officinalis (0.5%)	1.78 ± 0.39^{b}	1.50 ± 0.22^{a}	45.00 ± 0.71^{e}	44.89 ± 0.78^{d}	8.87± 0.31^{b}	8.74 ± 0.28^{c}
S. officinalis (1%)	1.47 ± 0.41^{ab}	1.45 ± 0.15^{a}	44.00 ± 0.87d^{e}	43.22 ± 0.97^{cd}	8.51± 0.39^{ab}	8.42 ± 0.41^{bc}
S. officinalis (1.5%)	1.38 ± 0.19^{a}	1.42 ± 0.19^{a}	43.22 ± 1.30^{cd}	43.55 ± 1.33^{cd}	8.37± 0.46^{ab}	8.36 ± 0.46^{bc}
A. vera (0.5%)	1.41 ± 0.20^{ab}	1.39 ± 0.17^{a}	42.44 ± 1.33^{abc}	42.11 ± 1.76^{abc}	8.04± 1.00^{ab}	7.87 ± 1.01^{b}
A. vera (1%)	1.30 ± 0.18a	1.44 ± 0.28^{a}	43.67 ± 0.71^{cde}	43.11 ± 1.05^{bc}	8.20 ± 0.41^{ab}	8.33 ± 0.43^{bc}
A. vera (1.5%)	1.33 ± 0.14^{a}	1.40 ± 0.14^{a}	43.00 ± 0.87^{bcd}	42.44 ± 1.51^{abc}	8.00± 0.51^{ab}	7.67 ± 0.49^{ab}

Data were subjected to analysis of variance (SPSS, One-Way ANOVA) followed by Tukey's test. The different superscript alphabets in the same column show significantly different groups at $p < 0.05$.

mortality compared to the control group and exhibited the least mortality among other treatments on days 30 and 45.

The histopathological results of gill tissue were shown some light focal and moderate multifocal lesions in the treatment groups. The lesions are exhibited in Figures 3 and 4. The intensity of the lesions in different groups is shown in Table 4.

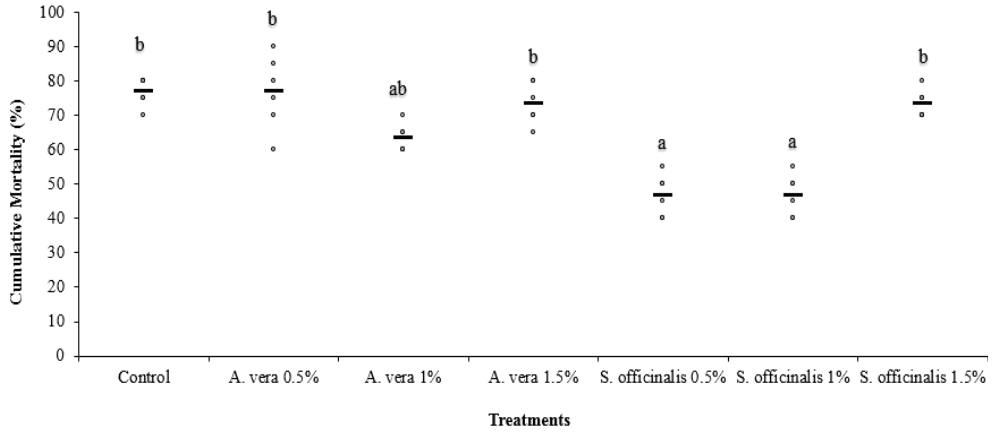

Figure 1.
Cumulative mortality of fish at the end of 6 hours expose to hypoxia stress at day 30. The different alphabets how significantly different groups ($p < 0.05$).

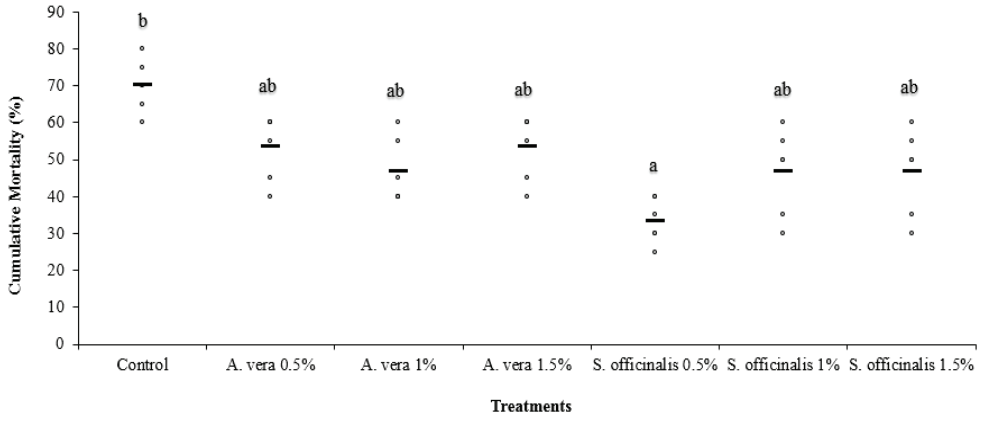

Figure 2.
Cumulative mortality of fish at the end of 6 hours expose to hypoxia stress at day 45. The different alphabets show significantly different groups ($p < 0.05$).

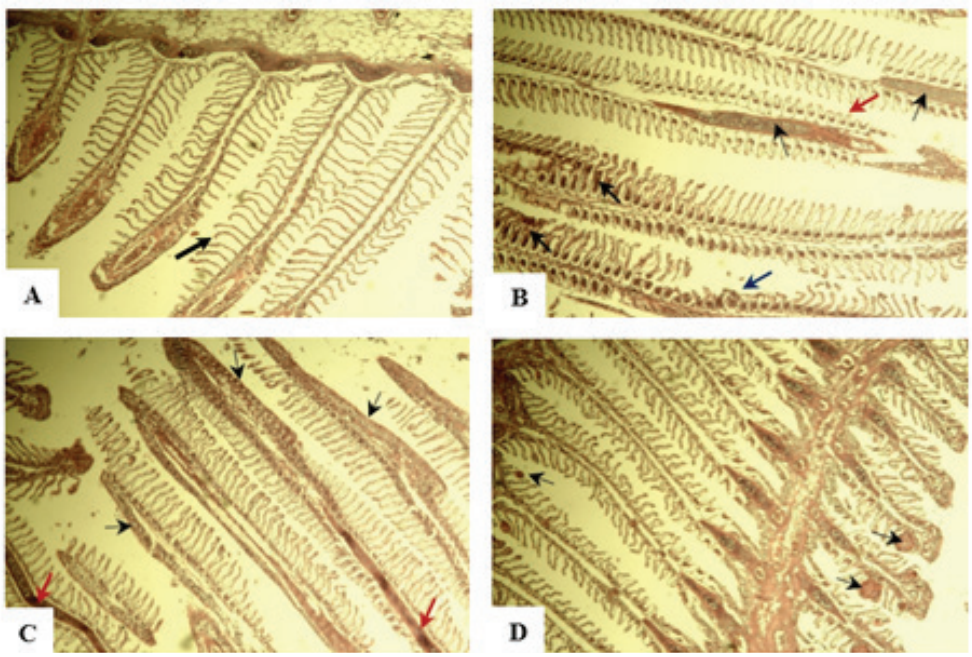

Figure 3.
Photomicrograph of the gill tissue of the control and A. vera groups at day 30 (H & E. ×100).

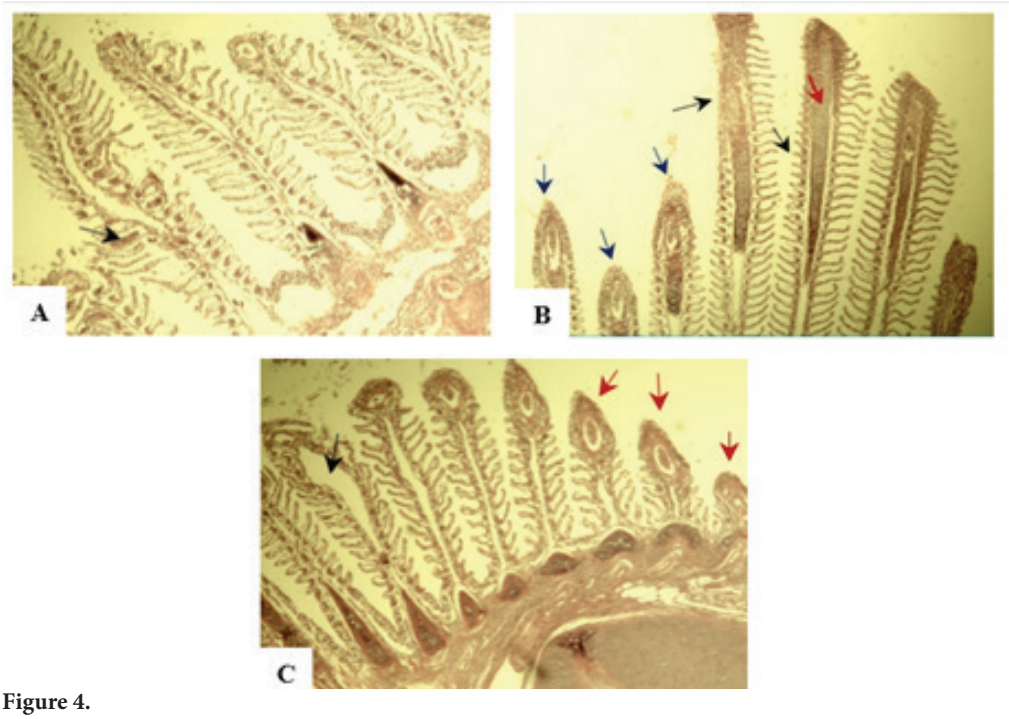

Figure 4.
Photomicrograph of the gill tissue of *S. officinalis* groups at day 30 (H & E. ×100).

Discussion

The *A. vera* and *S. officinalis* have been used in traditional medicine. These medicinal plants have good potential to be used as alternative growth promoter, antimicrobial, anti-stressor, and immunostimulator in fish [16, 17] and other animals such as poultry [18]. The beneficial properties of *A. vera* and *S. officinalis* are due to the presence of effective compounds in these plants (Table 1). Natural biogenic compounds of these medicinal plants were analyzed in the present study and some natural compounds such as Cineol, 1-Heptanol,2-propyl-, (4,7-Dinitronaphthalen-1-yl)-(4-methoxyphenyl) diazene, 1,2-Benzenedicarboxylic acid, diisooctyl ester followed by Squalene in *A. vera* and Cineol, Borneol, 1-Naphthaleneacetic acid, methyl ester, -Naphthalenol, 4-methoxy-1 and 1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (E)- in *S. officinalis* were identified as antimicrobial and immunostimulator agents in fish [19]. The Cineol is a monoterpenoid with anti-diabetic, antiviral, antispasmolytic, vermicide, poison antidote, antimicrobial, antifungal, free radical scavenging, antioxidant, neuroprotective and immune-stimulating effects that exists in both extracts in this study. This bio-active substance that affects some blood factors, make fish resistant to the environmental stress [19].

The current study demonstrated that inclusion of *A. vera* and *S. officinalis* extracts in *O. mykiss* diet markedly enhance hematological parameters. The results (Table 2) of total RBC count in *S. officinalis* (0.5%) treatment at day 30, hematocrit in *S. officinalis* (0.5%, 1% and 1.5%) treatments at days 30 and 45 and hemoglobin concentration in *S. officinalis* (0.5%) treatment at days 30 and 45 revealed significant differences compared to the control group ($p < 0.05$).

The *A. vera* (1% and 1.5%) treatments enhanced hematocrit at day 30 while hemoglobin concentration increased significantly in *A. vera* (0.5% and 1%) treatments compared to the control group at day 45 of the experiment (Table 2). Haghighi et al. (2014) reported no significant differences in total red blood cells, hematocrit and hemoglobin concentration between *A. vera* (1% of diet) treated group and the control group after eight weeks of feeding in the rainbow trout ($p < 0.05$) [20]. Some differences between their results and the results obtained by the current study might be related to the difference in fish age or extracts' composition. This difference can be attributed to the several environmental factors such as climatic, seasonal and geographical or ontogenesis variations, plant harvest time, error in the analysis of extracts and the time between extraction and analysis of compounds' constituents. No significant differences were observed in the red blood cell count between fish treated with any rate of *A. vera* and the control group (Table 2). These results are in consistent with the results obtained by other researchers [16, 21] who reported that common carp, *Cyprinus carpio* and rainbow trout treated with dietary *A. vera* supplementation had no significant differences in RBCs count [16, 21].

Some plasma chemicals may be useful indicators

Table 3
Glucose and cortisol of serum in day 30th and 45th, before and after hypoxic stress

Treatments	Glucose (mg dl^{-1})				Cortisol (ng ml^{-1})			
	Day 30		Day 45		Day 30		Day 45	
	Before Stress	After Stress	Before Stress	After Stress	Before Stress	After Stress	Before Stress	*After Stress*
Control	8.96 ± 1.20^{a}	12.07 ± 0.92^{a}	9.28 ± 0.60^{a}	12.7^{5} ± 0.57^{a}	12.36 ± 1.06^{a}	19.50 ± 1.68^{a}	12.50 ± 0.85^{a}	19.80 ± 1.43^{a}
S. officinalis (0.5%)	9.32 ± 0.40^{a}	15.15 ± 0.57^{b}	9.26 ± 0.29^{a}	13.33 ± 0.23^{a}	12.84 ± 1.19^{a}	28.84 ± 2.80^{b}	12.76 ± 2.00^{a}	21.94 ± 2.45^{a}
S. officinalis (1%)	8.50 ± 0.72^{a}	12.83 ± 1.71^{ab}	8.28 ± 1.08^{a}	12.53 ± 1.44^{a}	12.40 ± 0.47^{a}	24.17 ± 3.13^{ab}	12.10 ± 0.74^{a}	20.03 ± 1.21^{a}
S. officinalis (1.5%)	8.90 ± 0.22^{a}	12.92 ± 1.02^{ab}	8.55 ± 0.44^{a}	13.45 ± 0.71^{a}	12.61 ± 1.42^{a}	24.48 ± 3.58^{ab}	12.45 ± 0.23^{a}	20.65 ± 3.24^{a}
A. vera (0.5%)	9.13 ± 0.11^{a}	12.41 ± 0.48^{a}	8.76 ± 0.61^{a}	13.26 ± 0.48^{a}	12.47 ± 0.22^{a}	21.76 ± 3.74^{ab}	12.22 ± 0.87^{a}	20.61 ± 1.83^{a}
A. vera (1%)	8.78 ± 0.25^{a}	12.60 ± 0.48^{a}	8.98 ± 0.63^{a}	12.99 ± 0.58^{a}	12.35 ± 0.62^{a}	22.31 ± 3.52^{ab}	12.20 ± 0.94^{a}	20.69 ± 1.81^{a}

Data were subjected to analysis of variance (SPSS, One-Way ANOVA) followed by Tukey's test. The different superscript alphabets in the same column show significantly different groups at $p < 0.05$.

Table 4
Pathology scores of gill of fish given oral administration of *A. vera* and *S. officinalis* extract

Variables	Treatments						
	Controls	*A. vera* ***0.5%***	*A. vera* ***1%***	*A. vera* ***1.5%***	*S. officinalis* ***0.5%***	*S. officinalis 1%*	*S. officinalis* ***1.5%***
Atrophy of secondary lamellas	-[a]	+[b]	-	-	-	-	-
Degeneration and necrosis of lamellas	-	+	++[c]	-	-	++	-
Hyperemia	-	++	++	-	-	++	-
Clubbed secondary lamellas	-	-	-	++	+	-	-
Stunted gill filaments	-	-	-	-	-	++	++
Subepithelial space edema	-	-	-	-	-	-	+

a: No lesions, b: Light, focal lesions, c: Moderate, multifocal lesions

to evaluate the health and/or stress condition of the fishes [22]. Since stress has been reported to elevate plasma cortisol and glucose levels [23], many researchers consider as a "rule of thumb" that fishes undergoing stressful situations exhibit plasmatic increases of cortisol and glucose. Cortisol is the principal glucocorticoid secreted by the interrenal tissue (steroidogenic cells) located in the head-kidney of teleost fish. This hormone is released by the activation of the hypothalamus-pituitary-interrenal axis (HPI axis). When fish are exposed to stress, the hypothalamus releases corticotropin-releasing factor (CRF) polypeptide into the blood circulation. This factor affects the anterior pituitary gland and adrenocorticotrophic hormone (ACTH) releases into the blood circulation which ultimately activates the release of cortisol by the interrenal tissue [24]. Cortisol hormone function is to promote glycogenolysis and gluconeogenesis processes in fish. Another function of cortisol hormone is to induce the chromaffin cells to release catecholamines that further increases glycogenolysis and modulates the cardiovascular and respiratory functions. The goal of this chain of processes is to eventually produce enough glucose to provide the energy needed to cope with stress [24].

Two hours after the beginning of hypoxia stress period, *A. vera* had no significant effect on glucose and cortisol levels at any rate in this study (Table 3) and cumulative mortality of fishes in *A. vera* treatments did not significantly ($p < 0.05$) differ compared to the control group (Figures 1 and 2). Zanuzzo et al. (2012) reported that addition of *A. vera* (0.02, 0.2 and 2 mg/ml^{-1}) in the transport water did not affect the level of blood glucose of Brycon amazonicus [6]. Taiwo et al. (2005) demonstrated that Nile Tilapia, *Oreochromis niloticus* juveniles exposed to water containing 50, 100 and 150 mg/L^{-1} of the aqueous extract of *A. vera* leaves exhibited erratic swimming patterns, rapid opercular movements, skin depigmentation and died within 24-96 h [25]. Gross and histologic tissue lesions in the test fish include skin depigmentation, pale and shriveled gills, dull, opaque and sunken eyes, stunting and clubbing of gill filaments, vacuolar degeneration and necrosis of gill epithelial cells, hyaline degeneration and necrosis of myofibrils, calcification of vasa, hepatocellular vacuolar degeneration and necrosis. These authors explained that these complications might be emergent of high doses of applied *A. vera*. None of these complications were observed in the current study.

In most fishes, cortisol reaches the highest concentration 1 hour after being stressed and returns to basal levels after 6 hours [26]. Two hours after the beginning of hypoxia stress period, the glucose and cortisol levels in *S. officinalis* (0.5%) group was significantly higher than the control group at days 30 and 45 ($p < 0.05$), while other treatments didn't show the same effect. Moreover, *S. officinalis* (0.5%) group revealed significantly higher glucose levels compared to all rates of *A. vera* group at days 30 ($p < 0.05$) (Table 3). The increase of glucose level in the fish blood helps to satisfy the increased energy demand during stress, allowing the fish to react to stressors [6]. Hyperglycemia has been associated with stressful conditions such as hypoxia, capture, transport and crowding [27].

Cumulative fish mortality in *S. officinalis* (0.5% and 1%) treatment were significantly lower than the control group ($p < 0.05$) at the end of 6 hours hypoxia stress period at day 30 (Figures 1 and 2). The mortality rate was significantly lower only in *S. officinalis* (0.5%) treatment at day 45 compared to the control ($p < 0.05$) (Figure 2). Decreased mortality in *S. officinalis* treat-

ments is due to increase in total RBC, hematocrit, hemoglobin, glucose and cortisol levels which enhance the fish resistance towards hypoxia stress.

Taiwo et al. (2005) reported the *A. vera* as a toxic agent for tilapia and *O. niloticus* L. gills, but they consumed water containing the extract of raw *A. vera* leaves in static water, whereas in the current study hydroalcoholic *A. vera* and *S. officinalis* extracts supplemented diets were used for the rainbow trout [25]. Many researchers reported that *A. vera* and *S. officinalis* have benefits for histopathology of the liver [28], gonads [29] skin and gastrointestinal tract [17] of fish. In teh current study various focal and multifocal changes including atrophy of secondary lamellas, degeneration and necrosis of lamellas, hyperemia, clubbed secondary lamellas, stunted gill filaments and subepithelial space edema were observed in gills of treated fish with different plant extract concentrations (Figures 3 and 4). All lesions observed in the treatment groups in this study were either light or moderate and the comparison of lesions represented no significant differences between treatments (Table 4).

The results found in this study suggest that *A. vera* and *S. officinalis*, two medicinal plants extensively used for humans [5], are two promising natural products for aquaculture and should be more investigated as modulators of the hematological parameters and anti-stress agents in fish farming, since their use in the rainbow trout fingerlings improved its hematological and biochemical responses against hypoxia stress.

Material and methods

Plant extraction

Aerial organs of *A. vera* and *S. officinalis* were collected from local areas in Khuzestan Province of Iran and identified in the Department of Botany, Faculty of Agriculture, Urmia University, Iran. After identification, the plants were washed in running tap water to remove debris and dust particles and then rinsed in distilled water. Plant samples were air-dried and ground. Twenty grams of ground powders from each plant was soaked in 100 ml solvent (a mixture of ethanol: distilled water 50:50%) for 15 min with occasional shaking at 60 °C. After they were dissolved, the materials were filtered through Buchner funnel and Whatman No. 1 filter paper. Then, the filtrates were evaporated using rotary evaporator and concentrated.

Identification of extracts' components by GC/MS

The GC/MS analyses were performed on a Thermo Finnegan capillary gas chromatograph directly coupled to the mass spectrometer system (model GC TRACE; TRACE MS plus) using HP-5MS non- polar fused silica capillary column (30 m × 0.250 mm, 0.25 µm film thickness). Temperature profile was as follows: at first, the temperature of the oven was fixed at 40°C for 2 min, and then increased to 160 °C with the temperature rate of 3 °C min^{-1}, and finally increased to 280 °C at 5 °C min^{-1} for 2 min. The carrier gas was helium at a flow rate of 1 ml min^{-1}, and ionization energy was 70 eV [30].

Fish and husbandry conditions

In this study, 900 fish with a mean weight of 10 ± 0.1 g were obtained from a local farm in Urmia, Iran and transferred to "Artemia and Aquaculture Research Institute" of Urmia University. The fish were disinfected with 3% sodium chloride for 5 min and acclimatized to the laboratory conditions for a week. Fish were randomly distributed into 21 PVC tanks filled with 150 liters of dechlorinated fresh flow (3.5 L m^{-1}) water of a deep well. Water temperature and dissolved oxygen were 14 ± 1 °C and 8.5 mg L^{-1}, respectively.

Diet preparation and feeding trial

Extruded commercial fish feed (Faradaneh Co., Shahrekord, Iran) was used during the study with 90% dry matter, 38% crude protein, 16% crude lipid, 10% ash, 3% fiber and 1.20% phosphorous in its composition. Each herbal extract (*A. vera* and *S. officinalis*) was sprayed on the commercial diets at 0.5, 1, and 1.5% of diets separately. Pellets were dried at room temperature and were stored at 4°C for further use. Each diet was fed to triplicate tanks three times daily for a period of 30 days. After feeding trial, diets were replaced by a normal pelleted diet without any plant extract until day 45.

Assessment of hematological parameters

Fifteen fish from each treatment were anesthetized by immersion in a clove powder (200 mg L^{-1}) solution, and blood samples were collected from the caudal vein of fish [31], at days 30 and 45. The blood samples were mixed with heparin to determine red blood cells (RBC) count, hematocrit, and hemoglobin. The RBCs were counted under a light microscope using Neubauer hemocytometer after dilution with phosphate-saline. The cells were identified on the basis of morphology and cell ultra-structure as documented in a previous study [32]. The hematocrit percentage was determined through the microhematocrit method, and hemoglobin concentration was determined using cyanomethemoglobin method [33].

Histopathology procedures

Immediately after feeding for the trial period (day 30), the fish were anesthetized with immersion in a solution containing clove powder (200 mg L^{-1}) [31] and then sacrificed by cervical section. The gill samples were excised, rinsed in physiological saline, and fixed in aqueous Bouin's fluid for 6, 8 and 12 hours, respectively. The tissues were dehydrated in an ethyl alcohol series of ascending concentrations, embedded in paraffin and sectioned at 5 µm. The tissue sections were stained with hematoxylin-eosin (HE) and were examined by a light microscope [34].

The presence of histological alterations for gill was evaluated semi-quantitatively by the degree of tissue change that is based on the severity of the lesions. For degree of tissue change the alterations in each organ were classified in progressive stages of damage to the tissue: stage I alterations, which do not alter the normal functioning of the tissue; stage II, which are mild-severe the normal functioning of the tissue; and stage III, which are more severe and cause irreparable damage. A value of the degree of tissue change for each treatment was calculated by the below formula:

Degree of Tissue Change = (1 × stage I) + (10 × stage II) + (100 × stage III)

(Where I, II and III correspond to the number of alterations of stages I, II and III, respectively).

Degree of tissue change values between 0 and 10 indicate normal functioning of the organ; values between 11 and 20 indicate

slight damage to the organ; values between 21 and 50 indicate moderate changes in the organ; values between 50 and 100 indicate severe lesions and values above 100 indicate irreversible damage to the organ [34].

Hypoxia stress and biochemical indices assay

In hypoxia stress,, dissolved oxygen decreased to 3 mg L^{-1} in stress tanks (each tank contained 40-liter water) via infusion of nitrogen gas into water at days 30 and 45; then 30 fish of each treatment (10 fish from each replication) were exposed to hypoxia stress (3 mg L^{-1}) for 6 hours and mortality was recorded each hour. Then, fish of each group were placed in tanks containing 40 L of aerated flow-through well water. All fishes were monitored for 1 h and dead fish were removed and added to mortality data. Then, the mortality rate was recorded for each group.

The blood samples were collected from selected fish of each treatment (from the caudal vein after euthanasia of fish by clove powder 200 mg L^{-1}) [31] exactly before and one hour after hypoxia stress and transferred into Eppendorf tubes and allowed to clot at room temperature for 1 h. Then, samples were kept at 4 °C for 5 h. The serum was separated by centrifugation (1500 g for 5 min at 4 °C) and used for the glucose and cortisol levels estimation. The glucose and cortisol were quantified by electrochemiluminescence method using Mindray biochemical auto analyzer (BS - 400), with kits supplied by Mindray biomedical electronics Co. Ltd Shenzhen, China.

Statistical analysis

All data were subjected to analysis of variance (SPSS v20, One-Way ANOVA) followed by Tukey's test ($p < 0.05$). Figures and tables were drawn with office 2013.

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Author Contributions

Conceived and designed the experiments: A.A.T., S.M. Performed the experiments: A.A.T., A.T. M.A., F.N. Analyzed the data: A.A.T. Wrote the paper: A.A.T

Conflict of Interest

The authors declare no conflict of interest.

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Molecular detection of mouse hepatitis virus in laboratory mouse colonies

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ABSTRACT

The animal health monitoring is required to issue health certificates. The viral hepatitis virus is one of the most important infectious agents in mice breeding colonies. This research used RT-PCR to identify contaminations to mouse hepatitis virus. 18 out of 29 specimens were found to be infected, a prevalence of 62%. PCR product was purified and sequenced. Phylogenetic analysis revealed that the identified strain in this study was closely related to a strain reported from France. In the conventional system, contamination with different infectious agents is inevitable, thus it is better to replace the contaminated colonies with clean animals.

Keywords

Molecular detection, Mouse, Hepatitis virus

Abbreviations

MHV: Mouse hepatitis virus
ELISA: Enzyme linked immunosorbent assay
RT-PCR: Reverse transcription-polymerase chain reaction
NIH: National Institutes of Health
NC: Nucleocapsid
FELASA: Federation of European Laboratory Animal Science Associations
IVC: Individually ventilated cage

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Mouse hepatitis virus is a common infection in a large number of laboratory mouse colonies and is known to interfere with research results (1). MHV is an enveloped virus which has a 31Kb single-strand positive RNA genome. MHV belongs to the Coronaviridae family and replicates in the cytoplasm of infected cells using a viral RNA-dependent RNA polymerase which is translated from the genomic RNA (2, 3). MHV strains are classified as respiratory tropic or enterotropic groups based on tissue distribution of primary infection (2, 4), although the enterotropic infection is considered to be the most common from of infection (10). MHV is well known to be the most common virus of laboratory mice (2, 5). Natural infections with MHV remain widespread in most laboratory mouse populations despite the efforts to detect and eradicate this agent (6). Current data based on serological tests estimate that 60 to 80% of laboratory animal colonies are infected with MHV (2). Since its first description by Cheever in the late 1940's, MHV has been shown to alter the results of in vivo experiments using other infectious and non-infectious agents (2, 7). Concomitant infection with MHV has been correlated with altered responses to tumours (8) and to other viruses (2). Also, immune system-modulation experiments were noted to potentiate MHV infection and disease (2, 7). MHV is able to spread rapidly in mouse colonies because of its high contagiousness (2, 9, 10). Therefore an early detection of MHV infection is very important. Current methods which are used to detect MHV infection include ELISA and immunofluorescence techniques. The diagnosis of MHV infection is mainly performed by serological assays due to the difficulties in finding histological lesions and in isolating the virus in tissue culture (2, 11). However, the seroconversion of the animal sentinels or the newly infected ones requires a waiting period before a serologic assay can be used. The direct detection of viral nucleic acid using molecular bio1ogy methods in clinical or necropsy specimens would be a quick and powerful means to detect an outbreak or a sub-clinical condition affecting the animals (2, 11-14). RT-PCR has been effective in the detection of MHV in tissues and feces of infected mice (10, 12, 22). The aim of this study was to evaluate mouse hepatitis virus in NIH mice colonies in one laboratory animal facility in Iran using RT-PCR method.

According to the FELASA instruction, taking into account a 10% prevalence of contamination and 95% confidence, 29 samples was needed. In this study, NIH breeding mice from both sexes in the breeding room were randomly selected and monitored for mouse hepatitis virus according to the ethical protocols. Samples were collected from the intestine (colon) containing feces and prepared by standard methods. Then, RT-PCR was performed (10, 11, 12, 22). The sequence of NC gene (F: 5'- CAGCAGTGTTTTGGAAAGAGAG-3', R:5'- TGGGCTTTGCAACGCTTA-3')(2) available in the Genbank (Accession number, EMBL: AB551247.1) were cloned in pUC57 vector (CinnaGen, Tehran, Iran). The pUC57-NC plasmid was used as positive control. Plasmid extraction was performed by the GF-1 kit (Vivantis, Malaysia) in accordance with the protocol. RNA was extracted from intestinal tissue samples using Trizol (25). Contaminating genomic DNA was removed by DNase I (Fermentas, ... treatment) (25). The conversion of RNA to cDNA was carried out using the Viva 2 steps RT-PCR kit (Vivantis, Malaysia) (25). The reaction was carried out with a final volume of 25 µl according to the protocol (26). The PCR reaction ncluded: initial denaturation at 94 °C for 5 minutes, 30 cycles f denaturation at 94 °C for 1 minute, annealing at 52 °C for 1 minute, and extention at 72 °C for 1 minute, and a final extention at 72 °C for 10 minutes (26).

In 18 colon samples containing feces, the infection to MHV was positively detected. Therefore, the prevalence of this infection was calculated to be 62% (Figure 1).

For phylogenetic study, the positive sample was tested 3 times. Therefore, a PCR product was sequenced by BIONEER (South Korea). The alignment study was conducted through the EMBL-EBI and Klign (2.0) program and the sequence acquired from Sanger sequencing was compared with the sequences of the four other species obtained from the NCBI GenBank. In the phylogeny tree, the strain KX774640: 0.04601 belongs to this study and other species are X63538: 0.03507, L37760: 0.02799, L37759: 0.01186 and L37758: 0.02207, respectively. The most closely related strain in this study has been shown to be X63538: 0.03507 in France. The degree of affinity is found in the phylogeny tree (Figure 2).

Based on the recommendation of the FELASA, animal health monitoring is required to issue health certificates that are required for quality systems and quality control of production and research institutes (11, 15, 16). Many infectious agents in laboratory animals cause infections in humans and they are zoonoses (17, 18). Recommendations should be based on individual and local needs, considerations of research work, factors that are prevalent regionally, and national goals that are relevant in each country (11, 15, 16). The transmission of infectious agents and the presence of allergenic agents in open-cage systems are more prevalent than closed systems. Thus, it is much more important to carry out health monitoring programs in conventional open-cage systems. (11, 15, 16). In Iranian laboratory animal breeding centers, despite the advances made in design and breeding methods,

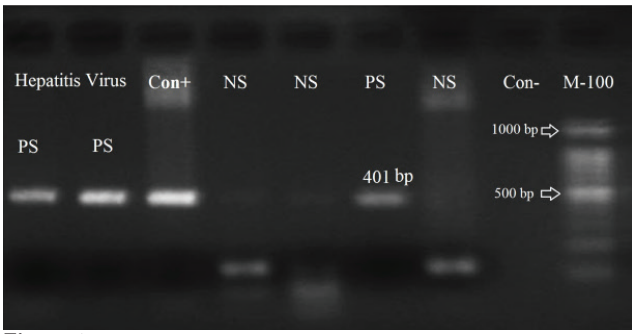

Figure 1
Agarose gel electrophoresis of RT-PCR products from Mouse Hepatitis Virus, NC gene. [M-100: PCRBIO Ladder IV DNA Marker- 100 bp (Arian Gene Gostar), Con-: Negative Control, Con+: Positive Control, NS: Negative Sample, PS: Positive Sample]

some infections, especially parasitic, bacterial and viral infections, are still present. Although clinical symptoms may not be seen in contamination with infectious agents, it can negatively affect the quality of the vaccine and biological products tested in these animals (11, 15).

In this study, the prevalence of this infection was 62%. Despite the advances made in the design and method of breeding centers especially in this center, the prevalence of this viral infection is still high. Although there are no clinical signs of contamination with these virus, it can negatively affect the results of the research and quality control tests. In the conventional system, contamination with different microbial agents is inevitable, but it is better to replace the contaminated colonies with clean animals. There are many reports on the health surveillance of viral infectious agents in foreign countries. The first description of the mouse hepatitis virus was provided by Cheever et al. (1949) (16). Parker (1979) identified 60-80% infection rates of mouse hepatitis virus in laboratory animals (17). Kagiyama et al. (1986) introduced the mouse hepatitis virus as one of the common viruses in laboratory mice (5). Homberger et al. (1991) and Yamada et al. (1993) introduced the RT-PCR as a suitable method for detecting the mouse hepatitis virus (18, 19). Yamada et al. (1993) announced that the virus could rapidly spread to the laboratory colonies due to easy transfer through contaminated materials (19). Adami et al. (1995)and Barthold and Smith (1990) published reports of viral hepatitis infection in mice and rats in both animal and wildlife animal breeding centers (3, 7). Jacoby and Lindsey (1997) reported the hepatitis mouse virus in 60% of the conventional breeding centers and 10% of the eligible systems of the barriers (20). Cecilio et al. (2000) detected mouse hepatitis virus by Nested PCR in liver tissue samples of laboratory mice (2). Matthaei et al. (1998) used the polymerase chain reaction to diagnose a natural outbreak of mouse hepatitis virus infection in nude mice (21). Oyanagi et al. (2004) detected the MHV-RNAs in mouse intestines and in filter dust in mouse room ventilation duct by a modified RT-nested PCR (22). Wang et al. (1999) diagnosed the mouse hepatitis virus contamination in nude mouse population by using RT-PCR (23). Nowadays, the large production and breeding centers are tested for the diagnosis of mouse hepatitis virus by PCR every six weeks (24). In Iran, there have been no investigations into this virus. Fallahi and Mansouri (2017) reported the health monitoring of NIH laboratory mice to Clostridium piliforme (24). The use of filter cages in the IVC breeding system prevents the transmission of airborne contamination. Although infection with the virus is unusual in humans, full compliance with health rules is required for staff working with rodents.

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Author Contributions

RF prepared the experimental design, wrote and revised the article, and managed the research. FA performed the experiments.

Figure 2
The phylogenic tree of the strain detected in this research (KX774640:0.04601) As shown in the picture, the most closely related specie is X63538: 0.03507 from France.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Case Report

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Severe subcutaneous, muscular and visceral coenurosis in a goat

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ABSTRACT

Coenurosis (gid or sturdy) is a zoonotic disease that is caused by Taenia multiceps metacestode. It is common in small ruminants. The cysts in sheep are more cerebral, while are noncerebral in goats. Coenurosis decreases production, and results in the death of the affected animals and in the disposal of the organs or even carcasses in severe infection. The present study describes severe subcutaneous coenuri associated with contamination in other tissues including the skeletal muscles and visceral organs. A remarkable clinical observation was the aggregation of cysts in variable sizes in the subcutaneous tissue of whole body. Subcutaneous tissue is not a common site for cyst formation. Coenurosis was confirmed based on the morphological characteristics of the cysts including the clusters of protoscolices and rostellar hooks.

Keywords

Coenurosis, Taenia multiceps, Coenurus cerebralis, goat

Abbreviations

C. gaigeri: Coenurus gaigeri
T. multiceps: Taenia multiceps
C. cerebralis: Coenurus cerebralis

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Introduction

Coenurosis (gid or sturdy) is a zoonotic disease that is caused by *Coenurus cerebralis* (*Taenia multiceps metacestode*). *Taenia multiceps* lives in the small intestines of carnivores as definitive hosts. Intermediate hosts are infected via ingestion of contaminated grass by spread eggs from the carnivores feces that lead to cyst formation in different organs [1]. Coenurosis is usual in small ruminants [2, 3], but rare in horses [4] and cattle [5]. The common predilection site for coenuri is cerebrum in sheep and extracerebral tissues in goats [1]. However, presence of cysts in the brain of goats [6] and other tissues apart from the brain of sheep [7] have been confirmed, recently. The parasite responsible for non-cerebral coenurosis was named *Coenurus gaigeri* in goats, and *Coenurus skrjabini* in sheep [1]. However, the later literature described *C. gaigeri* as the same species with *T. multiceps* [8].

Coenurosis causes high economic losses in the small ruminants industry and breeding [9]. Coenurosis decreases production, and in cases with severe infection leads to the death of the affected animals and disposal of organs or even carcasses [1, 10]. Human acts as incidental intermediate host and may be infected by ingestion of eggs in result of poor personal hygiene. In the literature, several reports of human coenurosis have been presented from different countries including Austria [11], Nigeria [12] and North America [13].

In the present study, we observed a lot of coenurus cysts in the subcutaneous, muscules and visceral organs of a goat.

Case presentation

A 11-month old female goat was referred to the veterinary hospital with a history of weight loss and multiple subcutaneous swellings on the face and around the eyes (Fig. 1a), neck, prescapular areas, flank and limbs (Fig. 1b). The case had not responded to any antibiotics or other treatments. On clinical examination, rectal temperature (38.5 °C), heart rate (75 per min) and respiratory rate (32 per min) were in the normal range. The subcutaneous palpable swellings were soft and fluctuating in different size from 2.1×3.4 to 8.5×10.2 cm. Clear watery fluid was aspirated by a steril syrige from the subcutaneous masses.

Collected cystic fluid had large number of small size, white colour plaques. The plaques were put on the clean glass slide, covered by a coverslip and examined under a light microscope. On microscopic examination, multiple protoscolices were observed. Based on the morphological features including the clusters of protoscolices and rostellar hooks (Fig. 2a), coenurosis was confirmed. Due to severe contaminataion, the goat was euthanized. In the postmortem investigation, Coenurus cysts were found under the skin (Fig. 2b), between fasciae of the skeletal muscles, in the thorasic cavity (Fig. 2c) and on the mesentery (Fig. 2d). The sizes of the coenuri cysts were different and had a thin and transparent wall. They were filled with clear fluid, and clusters of scolices were visible in their inner membrane (Fig. 2e).

Histopathologically, each coenurus was lined by a thin hyaline layer. Some cysts were surrounded by a demarcation line including lymphocytes, eosinophils, macrophages and giant cells. Several scoleces were visible within the cysts (Fig. 2f).

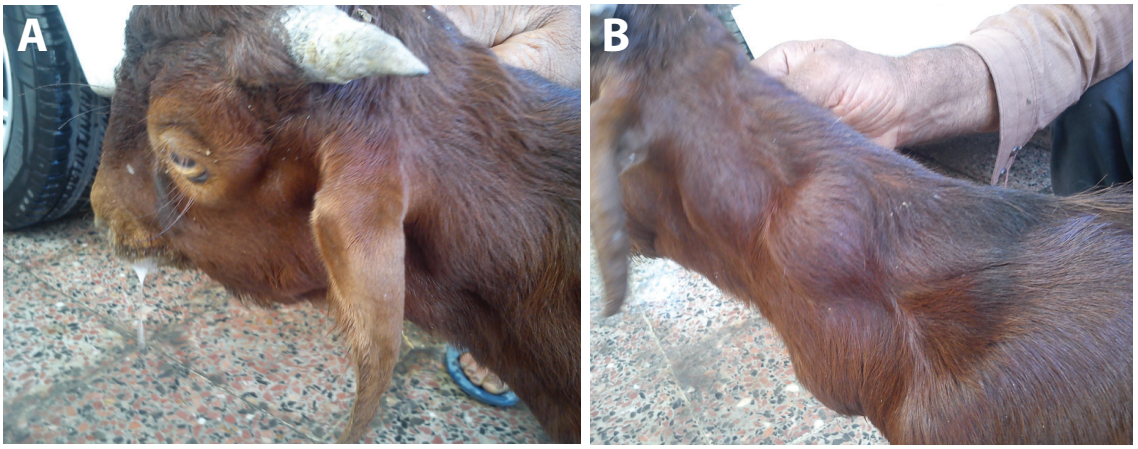

Figure 1
A: Goat affected to coenurosis. Different swellings on the face and periorbital region.
B: Coenurus cysts are located under the skin.

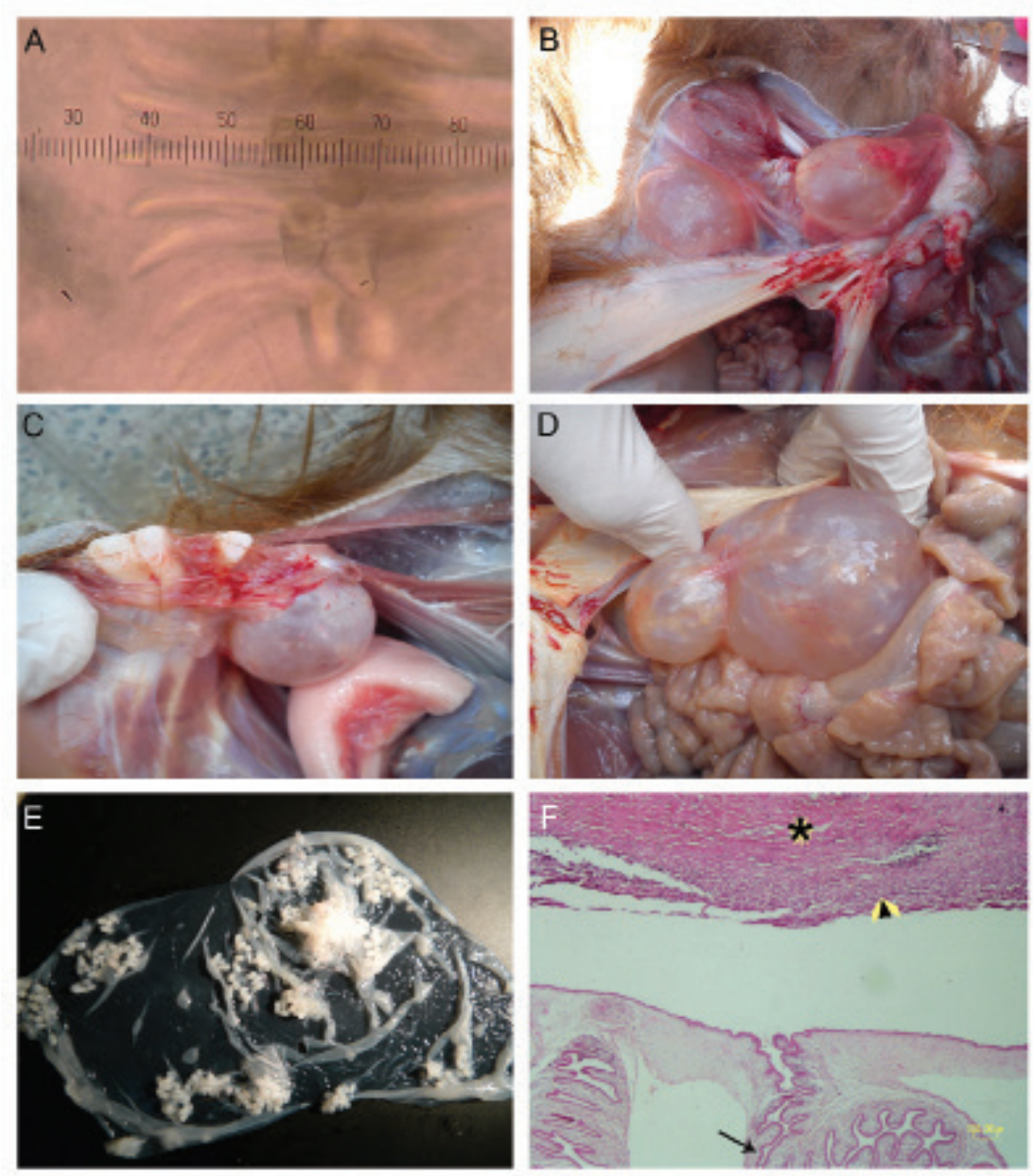

Figure 2
A) Large and small rostellar hooks, B) coenurus cysts are located under the skin, C) coenurus cyst attached to the costal muscles in the thoracic cavity, D) large coenuri on the intestinal mesentery, E) Isolated coenurus with seven clusters of protoscolices), F) photomicrograph shows a *Coenurus gaigeri* cyst in the skeletal muscles (asterisk). Capsule of cyst is composed of a dense outer hyaline layer and a disorganized inner layer. Multiple protoscolices (arrow) are observed within the cyst. Inflammatory cells (arrowhead) are infiltrated around the cyst.

All animals received human care in compliance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health, and the study was approved by the Ethical Committee of Shahid Bahonar Veterinary School (IR.UK.REC.1395.001).

Discussion

The occurrence of coenurosis in tissues other than CNS had been reported mainly from the Asian countries and are characterized to be *C. gaigeri* in goats [14, 15]. *C. cerebralis* and *T. multiceps* are considered the same species with only intraspecific variations.

Varcasia et al. (2012) investigated morphological and molecular characteristics of non-cerebral coenurosis in goats. They showed the same reported morphologic features with *C. cerebralis* reported by other authors [16]. The cysts outside of the CNS offer that a different strain or genetic variants of *T. multiceps* may be responsible. Phylogenetic trees based on genetic markers of mitochondrial DNA (ND1 and COI) demonstrated that non-cerebral cysts could belong to

different genotypes or strains of *T. multiceps*. Oryan et al (2010) evaluateted biochemical and pathological findings of *C. gaigeri* in Iranian native goats. They used CO1 and ND1 for phylogenetic analysis and identification of species. These researchers suggested that the larval stages of *T. multiceps gaigeri* and *C. cerebralis*, are monophyletic species [3]. According to the study of Hüttner et al. (2008), genetic analysis and phylogenetic investigation are the best diagnostic ways for identification of different species of metacestodes [17].

Clinical signs of coenurosis depend on the location and size of cysts [1]. Presence of cysts in the cerebrum is associated with the nervous symptoms including ataxia, paralysis, hypermetria, blindness, head deviation, incoordination, head pressing, and circling. Coenurosis may take for several months, and the mortality rate realted to that may reach to 100% [18, 19]. Non-cerebral coenurosis is not clinically diagnosable in mild form and the cysts may be observed in the slaughterhouse. In severe infection, the mainly clinical signs are lameness, paresis, paralysis and large skin lumps due to the subcutaneous cysts [20]. Muscular cysts cause pain and functional weakeness of involved organs [15]. Orbital coenurosis is rare and is associated with proptosis, blepharitis, the conjunctiva congestion, chemosis, swelling around the orbit, and enlargement and protrusion of the eye ball [20]. Treatment of coenurosis in sheep and goats with albendazole, niclosamide and praziquintal has little or no effect [1]. Surgical treatment for removing the cysts is not economical in cases with multiple large cysts.

In the present study, extra-cranial coenururi cysts affected the skeletal muscles, and subcutaneous and visceral organs. A remarkable clinical observation was aggregation of variable sizes cysts in the subcutaneous tissue of whole body. Our report described coenurosis in a 11-month female goat. It is stated that the disease happens often in 1-2 year-old female animals, particularly in the pregnancy course due to the pregnancy stress and reduction of immunity level. Previous studies show that clinical coenurosis is common in young animals [18, 22]. In the literature, there are reports similar to our report in goat [15]. Afonso et al. (2011) observed *C. cerebralis* in 149 abattoir-slaughtered and 47 experimentally infected goats. They showed that in the experimentally infected goats, a large percentage of *T. multiceps* cysts are found in the muscles and the subcutaneous tissues[23]. Shivapraksh and Reddy (2009) found multiple subcutaneous coenuri in the neck, prescapular region, abdomen and limbs in a herd of goats and characterized them as *C. gaigeri* due to their extra-cranial sites [2].

Coenurosis is a zoonotic pasitic disease and is important in public health. Human cysts are usually found in CNS, eye, subcutaneous or muscular tissues [24, 25, 26]. Control programs are regular anthelmintic treatment of dogs by effective taenicidal drugs, and correct disposal of contaminated carcasses to prevent access of dogs to them [27].

The present study reveals various predilection sites of coenurosis including the subcutaneous, skeletal muscles and other organs. Further studies are necessary to clarify the tendency of coenuri to occur in the subcutaneous tissues, skeletal muscles and visceral organs of goats. Public health importance should be considered in such cases. The awareness must be given to the farmers about the correct disposal of contaminated carcass. Regular antiparasitic drug should be used in dog for prevention of this parasitic infestation.

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Author Contributions

S.A. and R.Kh. performed post-mortem examinations. S.A. wrote the manuscript. M.A. referred the case and did the clinical examinations. SR.N. participated in the laboratory diagnosis of the cysts. All authors read and approved the final manuscript.

Conflict of Interest

All the authors declare that there is no conflict of interest.

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Abstracts (in Persian)

اثر سرما محافظی اریتریتول بر اسپرم منجمد قوچ

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چکیده

آزمایش برای بررسی جایگزینی گلیسرول با اریترتیول بر انجماد اسپرم قوچ انجام شد. نمونه منی از چهار قوچ در شش نوبت اخذ شد. در هر نوبت انزال ها تجمیع و بعد به ۱۲ بخش تقسیم شد. به هر بخش مقدار ۰.۰۳۲ مول گلیسرول (G۳۲E۰، برابر با ۳ ٪ گلیسرول)، ۰.۰۱۶ مول گلیسرول و ۰.۰۱۶ مول اریتریتول (۰.۰۰۸، G۱۶E۱۶ مول گلیسرول و ۰.۰۲۴ مول اریتریتول (۰.۰۳۲، G۸E۲۴) مول اریتریتول (۰.۰۵۴، G۰E۳۲ مول گلیسرول (G۵۴E۰، برابر با ۵ ٪ گلیسرول)، ۰.۰۲۷ مول گلیسرول و ۰.۰۲۷ مول اریتریتول (۰.۰۲۷، G۲۷E۲۷) مول گلیسرول و ۰.۰۴۱ مول اریتریتول (۰.۰۵۴، G۱۳E۴۱ مول اریتریتول (۰.۰۷۶، G۰E۵۴) مول گلیسرول (G۷۶E۰، برابر با ۷ ٪ گلیسرول)، ۰.۰۳۸ مول گلیسرول و ۰.۰۳۸ مول اریتریتول (۰.۰۱۹، G۳۸E۳۸ مول گلیسرول و ۰.۰۵۷ مول اریتریتول (G۱۹E۵۷) و ۰.۰۷۶ مول اریتریتول (G۰E۷۶) اضافه شد. نمونه های رقیق شده با روش استاندارد منجمد شدند. بعد از یخ گشایی نمونه به مدت شش ساعت در ۳۷ °C نگهداری شدند. نتایج نشان داد که پس از شش ساعت حرکت پیش رونده اسپرم و سلامت اکروزوم در تیمارهایی که فقط حاوی گلیسرول بودند کمتر از G۱۳E۴۱ (به ترتیب ۱۸.۸۵٪ و ۲۷.۴۱٪) بود ($p < ۰.۰۵$). در سطح ۰.۰۳۲ و ۰.۰۵۴ مول سرما محافظ، بیشترین تحرک اسپرم پس از شش ساعت به ترتیب در ۱۹.۱۶٪) G۸E۲۴) و ۱۸.۸۵٪ G۱۹E۴۱) مشاهد شد ($p < ۰.۰۵$). بنابراین کیفیت اسپرم منجمد قوچ را می توان با استفاده از ۰.۰۱۳ مول گلیسرول بعلاوه ۰.۰۴۱ مول اریتریتول یا ۰.۰۰۸ مول گلیسرول بعلاوه ۰.۰۲۴ مول اریتریتول بهبود داد.

واژگان کلیدی

انجماد، پلی یول، گلیسرول، اسپرم قوچ

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مطالعه مقایسه ای فعالیت لخته کنندگی ویتانیا کواگولانس در شیر گاو و مادیان

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چکیده

محدودیت های کاربرد رنین در تولید پنیر، اغلب سبب ترغیب به کشف پروتئازهای جدید می شود. میوه گیاه ویتانیا کواگولانس به دلیل فعالیت کازئینولیتیک، در تولید پنیر شناخته شده است. هدف از این مطالعه ارزیابی برخی از عوامل موثر بر فعالیت لخته کنندگی (MCA) عصاره آنزیمی میوه ویتانیا کواگولانس در شیر پستانداران زوج سم و تک سم است. عصاره ها با آب مقطر و نرمال سالین تهیه شده و محتوای پروتئین آنها بررسی شد. مدت زمان مورد نیاز برای پیدایش ذرات مجزای قابل تشخیص در شیر گاو و مادیان توسط دو غلظت از عصاره های نمکی و آبی ویتانیا کواگولانس (به ترتیب SE و HE) در حضور سطوح مختلف کلرید کلسیم و در دماهای ۳۵ و ۴۰ درجه سانتیگراد مورد ارزیابی قرارگرفت. برهم کنش این فاکتورها بر فعالیت لخته کنندگی شیر با استفاده از آنالیز واریانس طرح آمیخته ارزیابی شد. سه الگوی برهم کنش معنی دار که حاوی حداکثر تعداد فاکتور موثر بود، شناسایی شدند ($p < ۰/۰۵$). در این برهم کنش ها، غلظت بالای عصاره و دمای بالای انکوباسیون (۴۰ درجه سانتیگراد) همواره در ایجاد حداکثر فعالیت لخته کنندگی شیر موثر بودند. عصاره نمکی سریعتر از عصاره آبی لخته شیر را تشکیل داد. شیر گاو نسبت به شیر مادیان سوبسترای مناسب تری برای فعالیت آنزیم بود.

واژگان کلیدی

ویتانیا کواگولانس، فعالیت لخته کنندگی شیر، شیرگاو، شیرمادیان

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شیوع بالای گونه های پروتوتکا و جداسازی گونه های قارچی در نمونه های شیر از گاوهای مبتلا به ورم پستان در شهر مشهد، شمال شرق ایران

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چکیده

این مطالعه با هدف بررسی قارچ ها و جلبک های جدا شده از نمونه های شیر اخذ شده از گاوهای مبتلا به ورم پستان بالینی و تحت بالینی در گاوداری های اطراف مشهد انجام شد. ۵۰۳ نمونه ی شیر از ۱۰ گاوداری صنعتی گرفته و هر نمونه همزمان در محیط های بلاد آگار، مک کانکی آگار و سابورو دکستروز آگار حاوی کلرآمفنیکل کشت داده شد. تشخیص قارچ ها و جلبک ها با استفاده از خصوصیات فنوتیپی انجام شد. بیشترین میزان آلودگی مربوط به باکتری ها (۶۷.۲۰٪؛ ۳۳۸ نمونه از ۵۰۳ نمونه) بود و بعد از آن جلبک ها (۱۸.۵ ٪؛ ۹۳ نمونه از ۵۰۳ نمونه)، قارچ های رشته ای (۶.۴ ٪؛ ۳۲ نمونه از ۵۰۳ نمونه) و قارچ های مخمری (۵.۲ ٪؛ ۲۶ نمونه از ۵۰۳ نمونه) قرار داشتند. پنیسیلیوم (۲.۸ ٪)، آسپرژیلوس ها (۲.۶ ٪) ،کلادوسپوریوم (۱.۲ ٪)، ژئوتریکوم (۰.۴ ٪)، اولوکلادیوم، اسکوپولاریوپسیس و آلترناریا (هر کدام ۰.۲٪)، کریپتوکوکوس نئوفرمنس (۳ ٪)، کاندیداها (۱ ٪)، تریکوسپورون (۰.۸ ٪) و رودوتورولا (۰.۴ ٪) قارچ های رشته ای و مخمری جدا شده از نمونه های شیر مورد بررسی بودند. از ۹۳ جلبک جدا شده از ۵۰۳ نمونه شیر (۱۸.۵ ٪)، ۸۳ نمونه (۱۶.۵ ٪) آلوده به پروتوتکا زوپفی و ۱۰ نمونه (۲ ٪) آلوده به پروتوتکا ویکرهامی بودند. با توجه به نتایج حاصل از این تحقیق، قارچ های مخمری،کپکی و جلبک ها بخصوص پروتوتکاها از عوامل مهم آلوده کننده شیر بوده و در ایجاد ورم پستان نقش دارند. بنابراین، شیوه های بهداشتی مناسب و مدیریت گله های شیری و استفاده مدبرانه از آنتی بیوتیک ها برای کنترل آلودگی قارچی و جلبکی محیط و پستان ضروری است.

واژگان کلیدی

ورم پستان، گاو، قارچ ها، جلبک ها، گونه های پروتوتکا

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Abstracts (in Persian)

مقایسه اثرات محافظتی ال کارنیتین و عصاره آبی خار مریم پس از سمیت کبدی القا شده با دیازینون در کبد موش صحرایی نر

فروغ معصومی، مهرداد شریعتی، مختار مختاری

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چکیده

دیازینون (DZN) یک آفت کش ارگانوفسفور (OP)، با ایجاد استرس اکسیداتیو منجر به تولید رادیکال های آزاد و تغییرات پاتولوژیک در بدن است. هدف این مطالعه بررسی اثرات محافظتی ال کارنیتین (LC) و عصاره آبی خار مریم (SMAE) در برابر سمیت کبدی القا شده با DZN در کبد موش صحرایی نر است. موش ها در ۹ گروه (n=۸) شامل کنترل، شم، LC300 ، SMAE100 ، DZN15 DZN15 + SMAE100، DZN15 + LC300، SMAE100 + LC300 و DZN15 + SMAE100 + LC300 قرار گرفتند. ۳۰ روز پس از درمان تحت گاواژ دهانی، نمونه خون گرفته شد و سطح سرمی آسپارتات آمینوترانسفراز (AST)، آلانین آمینو ترانسفراز (ALT)، آلکالین فسفاتاز (ALP)، گاما گلوتامیل ترانسفراز (GGT)، آلبومین (Alb) و پروتئین تام (TP) با استفاده از روش فوتومتریک اندازه گیری شد. همچنین، کبد موش ها جدا گردید و مورد ارزیابی هیستوپاتولوژیک قرار گرفت. درمان با DZN15 به طور معناداری سطح AST، ALP، ALT و GGT را افزایش ($p < ۰.۰۵$) و برعکس، میزان Alb و TP را کاهش داد ($p < ۰.۰۵$). تجویز DZN۱۵ موجب التهاب لنفوسیتی، پرخونی، آپوپتوزیس هپاتوسیت ها و کوچکتر شدن فضای سینوزوئیدی شد. با این حال، درمان با DZN15 + SMAE100 LC300 سطح Alb، GGT، ALP، ALT و TP را تغییر نداد ($p < ۰.۰۵$)، اما سطح AST را به میزان قابل توجهی نسبت به گروه کنترل افزایش داد ($p < ۰.۰۵$). همچنین، تغییرات ساختاری کمتر و بهبود در بافت کبد مشاهده شد. یافته های این مطالعه نشان می دهد که تجویز همزمان SMAE و LC می تواند آسیب های بافت کبدی القا شده با DZN را کاهش دهد و پارامترهای بیوشیمیایی کبدی را در موش های صحرایی بهبود بخشد.

واژگان کلیدی

دیازینون، ال کارنیتین، خار مریم، سمیت کبدی، موش صحرایی

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Abstracts (in Persian)

بررسی تاثیر مکمل سین‌بیوتیکی لاکتوباسیلوس پنتوسئوس و لاکتوباسیلوس پلانتاروم به‌همراه بتاگلوکان بر فاکتورهای ایمنی ذاتی و فلور باکتریایی دستگاه گوارش قزل‌آلای رنگین‌کمان (*Oncorhynchus mykiss*)

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چکیده

در مطالعه حاضر، تاثیر پروبیوتیک باکتری های درون زاد لاکتوباسیلوس پلانتاروم و لاکتوباسیلوس پنتوسئوس به تنهایی یا در ترکیب با ۱و ۳ بتاگلوکان ۱ ٪ که یک کاندید پری¬بیوتیکی جدید است، در قزل آلای رنگین کمان جوان مورد بررسی قرار گرفت. این آزمایش در ۸ تیمار طراحی شد: غذای بدون مکمل یا غذای پایه، حاوی یک درصد بتاگلوکان در هر گرم خوراک، لاکتوباسیلوس پلانتاروم CFU/ ۱۰۸ gr، پنتوسئوس، پلانتاروم و پنتوسئوس به نسبت مساوی، پلانتاروم و یک درصد بتاگلوکان، پنتوسئوس و یک درصد بتاگلوکان، پلانتاروم و پنتوسئوس به نسبت مساوی یک درصد بتاگلوکان. برای هر تیمار ۶۰ قطعه بچه ماهی قزل¬آلا به وزن ۲±۱۵ در مخازن ۳۰۰ لیتری تقسیم‌بندی شد. بعد از ۸ هفته پاسخ ایمنی ذاتی ماهیان تا حدودی در ترکیب بتاگلوکان و پروبیوتیک افزایش یافت. اگرچه اثر سینرژیسم بتاگلوکان و پروبیوتیک بر آنتی تریپسین، باکتری کشی و انفجار تنفسی تنها در ماهیان تغذیه شده با جیره غذایی ۴ و ۶ مشاهده گردید، اما در سایر پاسخ های ایمنی هومورال در گروه های پروبیوتیکی، گروه پروبیوتیک- سین بیوتیک اختلاف معنی داری با گروه کنترل نداشت. بررسی میکروفلور دستگاه گوارش نشان داد که بتاگلوکان استقرار و ظرفیت رشد دو گونه باکتریایی درون زاد را بهبود بخشید. فراوانی نسبی باکتری های اسید لاکتیک در ماهیان با جیره غذایی لاکتوباسیلوس پلانتاروم و لاکتوباسیلوس پنتوسئوس به همراه بتاگلوکاگون بیش تر بود و توانست باعث تغییرات معنی داری را در میکروفلور روده ها به واسطه کاهش کل باکتری ها نسبت به گروه کنترل شود همچنین سبب تقویت سیستم ایمنی در ماهیان قزل آلای جوان شد.

واژگان کلیدی

۱و۳ بتاگلوکان، پاسخ ایمنی، پروبیوتیک، سین‌بیوتیک، قزل‌آلای رنگین‌کمان

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تاثیر جیره غذایی حاوی عصاره آلوئه ورا و مریم گلی بر خون‌شناسی، آسیب‌شناسی بافتی و مقاومت در برابر شرایط کم‌اکسیژنی در قزل‌آلای رنگین‌کمان

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چکیده

این مطالعه به بررسی آثار عصارهای آلوئه ورا و مریم‌گلی روی شاخص‌های خون‌شناسی و بیوشیمیایی، آسیب‌شناسی بافتی و مقاومت در برابر تَنِش کم‌اکسیژنی در قزل‌آلای رنگین‌کمان پرداخته است. هفت تیمار از قزل‌آلاهای رنگین‌کمان (۱۰ ± ۰/۱ g) با مقادیر ۰ (گروه شاهد)، ۰.۵٪، ۱٪ و ۱.۵٪ از عصاره‌های آلوئه ورا و مریم‌گلی به صورت ترکیب با غذای آنها به مدت ۳۰ روز تغذیه شدند و سپس به مدت دو هفته دیگر تغذیه همه ماهیان با غذای گروه شاهد ادامه یافت. در روزهای ۳۰ و ۴۵ نمونه‌های خون ماهیان گرفته و تعداد کل گلبول‌های قرمز، هماتوکریت و هموگلوبین اندازه‌گیری شد. در روزهای ۳۰ و ۴۵ گلوکز و کورتیزول سِرُم قبل و یک ساعت بعد از اِعمال تَنِش کم اکسیژنی ($mg\ L^{-1}$) اندازه‌گیری شد. در انتهای آزمایش نمونه‌های بافت آبشش همه تیمارها برای آسیب‌شناسی‌بافتی تهیه شد. تیمار مریم‌گلی ۰.۵٪ افزایش معنی‌دار ($p < ۰.۰۵$) در تعداد گلبول‌های قرمز، هماتوکریت و هموگلوبین را در مقایسه با گروه شاهد نشان داد. در حالیکه تیمارهای آلوئه ورای ۱٪ و ۱.۵٪ در مقایسه با گروه شاهد افزایش معنی‌دار ($p <۰.۰۵$) هماتوکریت و غلظت هموگلوبین را نشان دادند. پس از اِعمال تَنِش کم‌اکسیژنی در روزهای ۳۰ و ۴۵، سطوح گلوکز و کورتیزول سِرُم فقط در تیمار مریم‌گلی ۰.۵٪ در مقایسه با گروه شاهد بطور معنی‌داری ($p < ۰.۰۵$) افزایش یافت. در تیمارها و گروه کنترل تغییرات آسیب‌شناسی بافتی جدی مشاهده نشد.. بر اساس نتایج به دست آمده، تجویز خوراکی ۰.۵٪ عصاره آبی اتانولی مریم‌گلی موجب بهبود شاخص‌های خونی و بیوشیمیایی خون شده و مقاومت قزل‌آلای رنگین‌کمان را در برابر تَنِش کم‌اکسیژنی افزایش می دهد.

واژگان کلیدی

قزل‌آلای رنگین‌کمان، عصاره‌های گیاهی، تَنِش های محیطی، خون‌شناسی، آسیب شناسی بافتی

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Abstracts (in Persian)

تشخیص مولکولی ویروس هپاتیت موشی در کلنی موش های آزمایشگاهی

روزبه فلاحی[1]، فاطمه عابدینی[1]، غلامرضا شکری[2]

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چکیده

پایش بهداشتی حیوانات، جهت صدور گواهی سلامت آنها الزامی است. ویروس هپاتیت موشی از مهمترین عوامل عفونی در کلنی موش های آزمایشگاهی می باشد. روش تحقیق در این بررسی، RT-PCR ، با استفاده از پرایمرهای اختصاصی ویروس مورد نظر بود. تعداد ۱۸ نمونه از ۲۹ نمونه از موش ها، مبتلا به ویروس هپاتیت موشی، با میزان شیوع ٪۶۲ بوده اند. محصول PCR انجام شده تخلیص و تعیین توالی گردید. در درخت فیلوژنی، سویه متعلق به این تحقیق بیشترین قرابت را، با سویه ای از کشور فرانسه نشان داد. در سیستم های پرورش متعارفی، آلودگی با عوامل مختلف میکروبی اجتناب ناپذیر است، بنابراین بهتر است حیوانات پاک با کلنی های آلوده جایگزین شوند.

واژگان کلیدی

تشخیص مولکولی، موش، ویروس هپاتیت

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سنروزیس شدید زیرجلدی، عضلانی و احشایی در یک بز

شهرزاد عزیزی[1]، مهدی امیرمحمدی[2]،رضا خیراندیش[1]، سعید رضا نوراللهی فرد[1]

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چکیده

سنروزیس نوعی بیماری زئونوز است که توسط مرحله لاروی تنیا مولتی سپس ایجاد می شود. در نشخوارکنندگان کوچک رایج است. در گوسفند، کیست ها بیشتر در مغز و در بز در بافت های دیگر تشکیل می شود. سنروزیس سبب کاهش تولید، مرگ، دفع اندام ها و یا حتی لاشه در عفونت های شدید می شود. مطالعه حاضر، آلودگی شدید بافت های زیرپوست، عضلات اسکلتی و احشاء را به کیست های سنروس در بز توضیح می دهد. مهمترین نشانه بالینی قابل توجه وجود کیست های فراوان در اندازه های متغیر در زیر پوست است. مورفولوژیکی از جمله گروه های متعدد پروتواسکولکس در دیواره کیست و قلاب های حلقوی کیست سونوروزیس را تایید می کند. اگرچه بافت های زیر جلدی یکی از مکان های تشکیل کیست این انگل است با این وجود آلودگی های شدید مشابه گزارش مورد نظر رایج نیست. با این وجود، بافت زیر جلدی، محل تشکیل کیست است که معمول نیست.

واژگان کلیدی

لتی سپس، تنیام، زیس، سنر

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Guide for Authors

IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

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Guide for Authors

Acknowledgements

Personal acknowledgement, sources of financial support, contributions and helps of other researchers and everything that does not justify authorship should be mentioned in this section, if required.

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Conflict of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts
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Introduction should be as concise as possible, and clearly explain the main objective and hypothesis of the investigation.

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Results indicate the results of an original research
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Discussion should include the answer to the question proposed in the introduction and emphasize the new and important aspects of the study and the conclusions that follow from them. It could include the implication, application, or speculation of the findings and their limitations, relate the observations to other relevant studies, and links the conclusions with the goals of the study. Recommendations, when appropriate, may be included.

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Materials and methods should be described in sufficient details to allow other workers to reproduce the results. Specify any statistical computer programs used .The methods of data collection and use of statistical analysis will be checked by

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Example piece of text and reference list :

An unhealthy diet, obesity and physical inactivity play a role in the onset of type 2 diabetes, but it has been shown that increased physical activity substantially reduces the risk [1], and participation in regular physical activity is one of the major recommendation of the evidence based guidelines for the primary prevention of diseases [2]. According to the 2004-05 National Health Survey, more than half a million Australians (3.5% of the population) have diabetes mellitus which had been medically diagnosed and most of these people have the Type 2 condition [3]. Gestational diabetes is also on the increase, rising steadily between 2000-01 and 2005-06 [4]. Approximately two thirds of those with diabetes have been prescribed medication [3], but it is of concern that a recent review of the literature found that many people do not take their medication as prescribed [5]. Many patients also self monitor the disease by measuring their blood glucose levels with a glucose meter but Song and Lipman [6] have concerns about how well this is managed.

References for the above example:

1. Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015.

2. Eckerman AK, Dowd T, Chong E, Nixon L, Gray R, Johnson S. Binan goonj: bridging cultures in Aboriginal health. 3rd ed. Chatswood, NSW: Elsevier Australia; 2010.

3. Johnson C, Anderson SR, Dallimore J, Winser S, Warrell D, Imray C, et al. Oxford handbook of expedition and wilderness medicine. Oxford: Oxford University Press; 2015.

4. McLatchie GR, Borley NR, Chikwe J, editors. Oxford handbook of clinical surgery. Oxford: Oxford University Press; 2013.

5. Petitti DB, Crooks VC, Buckwalter JG, Chiu V. Blood pressure levels before dementia. Arch Neurol. 2005 Jan;62(1):112-6.

6. Liaw S, Hasan I, Wade, V, Canalese R, Kelaher M, Lau P, et al. Improving cultural respect to improve Aboriginal health in general practice: a multi-perspective pragmatic study. Aust Fam Physician. 2015;44(6):387-92.

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