

Original article

## ***In vitro* changes in adaptive cell-mediated immunity under medicinal plant extract treatment in resting horses from different workout backgrounds**

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### Abstract

The study aimed at investigating the *in vitro* immunological efficacy of various alcoholic plant extracts in horses from different workout regimens (semi-intensive and extensive) during their sitting period in connection to their carried microbiome. The *in vitro* blast transformation assay was carried out on heparinized blood samples from draft horses and sports horses during their sitting period. Cultures were performed using alcoholic extracts of *Sylibum marianum*, *Vaccinium myrtillus*, *Thymus vulgaris*, *Aloe vera* and *Hippophaer hamnoides*. At the end of incubation (48 h), the cell growth was estimated by a glucose consumption assay. Microbial samples were taken simultaneously and subjected to classical methods to identify the components of the carried microbiome. Student's 't' test was used to estimate the significance of the differences. A wide range of bacteria were identified from the respiratory system of both horse categories, with some differences between the workout groups. Statistically non-significant but stronger spontaneous and plant extract induced blastogenic indices were obtained for the semi-intensively exploited horses. The responses to *S. marianum* ( $82.2 \pm 18.74\%$ ) and *A. vera* ( $80.72 \pm 18.74\%$ ) in sports and draft horses, respectively were the highest. The other extracts exerted an inhibiting effect, mainly in the draft horses. The results indicated that, in spite of the sitting period, the workout background not only influenced the carried microflora but also the cell-mediated response to plant extract stimulation, thus potential immune stimulating and antibacterial treatments should be designed, based on the workout and also the productive period.

**Key words:** Horses, workout background, medicinal plants, adaptive immunity, stimulation

### 1. Introduction

The active life of a horse involves permanent contact with physical, chemical or biological stressors inducing changes, sometimes clinically noticeable at the level of one of the most sensitive sensors of the body, the immune system. The dysfunction of immune system is linked in the pathophysiology and aetiology of numerous disorders (Subramoniam *et al.*, 2013; Ahmad *et al.*, 2015). Monitoring such changes may indicate the animal's health status and provide useful information for the breeder, user or sportsman, which can thus take the optimal measures to ensure the welfare of the animal. The adjustment of immune responses using natural products has been of a great interest for several years. Given their enteral availability and their volatile property, the essential oils are currently used as food additives (Tisser and Young, 2014; Huerta *et al.*, 2016). Herbal drugs possess immunomodulatory and antioxidant properties and the mechanism of action implies suppressing or stimulating both specific and nonspecific immunities

(Ahmad *et al.*, 2015; Pandey *et al.*, 2006; Desalegn, 2014). Current conventional medicine uses many chemicals derived from herbs as therapeutic agents (Iqbal, 2013; Biradar, 2015; Dang, 2018). Recently, alternative medicines have gained consideration in the treatment of several immune diseases (Desalegn, 2014). Medicinal plant extracts used for immunomodulation can provide potential replacements to conventional chemotherapies for a variety of diseases, particularly in the case of impaired immune response (Ahmad *et al.*, 2015). Our study aimed at investigating the *in vitro* immunological efficacy of various alcoholic plant extracts in horses from different workout regimens (semi-intensive and extensive) during their sitting period in connection to their carried microbiome.

### 2. Materials and Methods

#### 2.1 Animals

The study design was carried out in agreement with the rules and standards set out by the Bioethics Commission of the Faculty of Veterinary Medicine, Cluj-Napoca. Blood samples collected from horses raised in extensive (n=8) and *semi-intensive* system (n=20) were subject to *in vitro* testing for their blast transformation capacity (glucose consumption test). The tested animals were clinically healthy at the moment of sampling. Blood from jugular vein (5 ml /50 IU heparin/ml) was collected from each animal before the morning feeding. Blood samples were processed within 1h of

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collection. Stimulation indices (%) were calculated compared to a glucose control. Also oral (n=28) swabs were collected individually horses. The samples were subjected to classical methods to identify the components of the carried microbiome.

## 2.2 Plant extracts

Commercial alcoholic extracts of *S. marianum*, *V. myrtillus*, *T. vulgaris*, *H. rhamnoides* (Plant extract, SRL Romania) and *Aloe vera* (Secom, Romania) produced according to the German Homeopathic Pharmacopeia, were used to treat the whole blood cultures. The identification of the plant species was carried out by Professor M. Tamas, Department of Pharmaceutical Botany, University of Medicine and Pharmacy, Cluj-Napoca, Romania and Plant extract, SRL Romania. The concentration of plant extracts used in our experiments were ascertained in pilot studies.

## 2.3 Glucose consumption test

In our experiment for evaluation of blast transformation capacity of the cells, glucose consumption tests were used, according to the protocol described by Khokhlova *et al.* (2004). The samples were diluted with RPMI 1640 (Sigma-Aldrich, USA) medium (1:4) with 5% FCS (Gibco) and penicillin and streptomycin (Sigma-Aldrich). The diluted samples were added 96-well plate, 100 µl/well, in duplicate, 8 variants: (1) untreated control culture, (2) phytohaemagglutinin-M (PHA) (1 µl/well) treated culture, (3) phytohaemagglutinin-M (PHA) (2 µl/well) treated culture, (4) phytohaemagglutinin-M (PHA) (5 µl/well) treated culture, (5) lipopolysaccharide (LPS) (1 µl/well) treated culture, (6) lipopolysaccharide (LPS) (2 µl/well) treated culture, (7) lipopolysaccharide (LPS) (5 µl/well) treated culture, (8) concanavalin A (Con A) (1 µl/well) treated culture, (9) concanavalin A (Con A) (2 µl/well) treated culture, (10) concanavalin A (Con A) (1 µl/well) treated culture, (3) alcohol (1.5 µl/well) treated culture and (4) alcoholic extracts of *S. marianum* (1.5 µl/well) treated culture, (5) alcoholic extracts of *V. myrtillus* (1.5 µl/well) treated culture, (6) alcoholic extracts of *T. vulgaris* (1.5 µl/well) treated culture (7) alcoholic extracts of *H. rhamnoides* (1.5 µl/well) treated culture and (8) extract of *A. vera* (1.5 µl/well) treated culture. The cultures were incubated for 48 h at 37°C and 5% CO<sub>2</sub>. Glucose concentrations were measured in the initial medium and in all variants, using orto-toluidine colorimetric test. 12.5 µl of the cultural medium was transferred to 0.5 ml of orto-toluidine reagent, heated for 8 min, suddenly cooled in cold water and evaluated using a spectrophotometer at 610 nm wavelength (Sumal PE2, Karl Zeiss, Germany), using the reagent as a blank. For transformation index (TI), the following formula was used:  $TI \% = [(MG - SG) / MG] \times 100$ , where TI, blast transformation index, MG, glucose concentration in the initial culture medium and SG, glucose concentration in the sample after incubation.

## 2.4 Statistical analysis

For statistical interpretation, Minitab 16.0 were used. Results were expressed as Mean ± standard deviation.

## 3. Results and Discussion

A wide range of bacteria were identified from the respiratory system of both horse categories, with some differences between the workout groups. The ported microbiome in resting horses from semi-intensively exploited system were *Staphylococcus intermedius*

(n=6), *Staphylococcus lentus* (n=6), *Streptococcus spp.* (n=12), *Corynebacterium spp.* (n=11), *Cellulomonas spp.* (n=6), *Pasteurella spp.* (n=6), *Neisseria* (n=5), *Bacillus necrophorus* (n=2), *Klebsiella spp.* (n=5), *Proteus spp.* (n=2), *Clostridium spp.* (n=5), *Bacteroides spp.* (n=2), *Aerococcus viridans* (n=2). In extensive system, the isolated and identified strains were: *Staphylococcus intermedius* (n=2), *Staphylococcus lentus* (n=4), *Streptococcus spp.* (n=2), *Corynebacterium spp.* (n=4), *Cellulomonas spp.* (n=2), *Pasteurella spp.* (n=4), *Neisseria* (n=1), *Bacillus necrophorus* (n=1), *Klebsiella spp.* (n=1), *Clostridium spp.* (n=1), *Micrococcus 25%* (n=1). Following the blast transformation test, stimulation / inhibition indices were calculated. The results obtained are presented in Table 1.

**Table 1:** Results obtained in the blast transformation test

	Semi-intensivesystem							
	CTRL (%)	PHA M (%)	AL (%)	Sybilum (%)	Hippophae (%)	Vaccinium (%)	Thymus (%)	Aloe (%)
Aver.	78.88	82.85	78.18	82.20	78.97	75.19	73.04	81.54
St.Dev.	84.97	93.46	84.75	89.36	92.59	86.58	89.86	93.85
	Extensive system							
	CTRL (%)	PHA M (%)	AL (%)	Sybilum (%)	Hippophae (%)	Vaccinium (%)	Thymus (%)	Aloe (%)
Aver.	75.47	74.53	78.27	64.25	75.23	67.64	65.30	80.72
St.Dev.	91.81	89.60	93.36	89.64	92.88	90.64	82.19	90.56

CTRL - control culture, PHAM = phytohaemagglutinin-M treated culture, AL = Alcohol 70°C treated culture, Sybilum = culture treated with alcoholic extract of *S. maritimum*; Hippophae = culture treated with alcoholic extract of *Hippophae rhamnoides*; Vaccinium = culture treated with alcoholic extract of *Vaccinium myrtillus*; Thymus = culture treated with alcoholic extract of *T. vulgaris*, Aloe = culture treated with *A. vera* extract.

Both human and veterinary medicine have used medicinal plants as therapeutic means, alone or in combination to prevent or treat diseases but also as nutritious supplements (Ansari, 2016). In the samples from extensive system were remarked an inhibitory effect of vegetal extracts, except for the alcoholic extract of *A. vera* which has a stimulating effect. Were also noted the slightly stimulatory effect of alcohol compared to untreated control. Comparing the results from the blast transformation capacity of cells in samples harvested from semi-intensively exploited horses, we noticed an inhibitory effect of the alcohol against the control group, while the vegetal extracts have variable effects depending on the extract.

Statistically, non-significant but stronger spontaneous and plant extract induced blastogenic indices were obtained for the semi-intensively exploited horses. The responses to *S. marianum* (82.2 ± 18.74%) and *A. vera* (80.72 ± 18.74%) in sports and draft horses, respectively were the highest. The other extracts exerted an inhibiting effect, mainly in the draft horses. The results indicated that, in spite of the sitting period, the workout background not only influenced the carried microflora but also the cell-mediated response to plant extract stimulation, thus potential immune stimulating and antibacterial treatments should be designed based on workout and also the productive period.

The immune system is deeply involved in the process of adapting the organism to environmental conditions, due to its role of protecting against environmental aggressors and its inter-relationship with the nervous system and the endocrine system. Corticosteroid hypersecretion, occurring as a defense response to microclimate conditions and its alterations, induces the involution of lymphoid tissue and depresses inflammatory reactions, including phagocytosis,

having similar effects also on cell mediated specific immune response (Arkin *et al.*, 1991). Monocytes and neutrophils are the first line of defence against pathogens, play a key role in innate immunity (Janeway *et al.*, 2011; Robson *et al.*, 2003), but adaptive immune system offer a more complex defence, with enhanced protection against succeeding reinfection (Janeway *et al.*, 2011). For the specificity of adaptive immune responses are responsible the lymphocytes, which generally respond to foreign antigens only if the innate immune system is first activated (Alberts *et al.*, 2002). Strenuous exercise suppresses the function of these phagocytic cells (Robson *et al.*, 2003; Raidal *et al.*, 2000). Prolonged and also short-term high intensity exercises induce a prolonged suppression of phagocyte function in horses (Raidal *et al.*, 2000; Krumrych *et al.*, 2018). After prolonged exercise may occur significant leucocyte and hormonal perturbations and a long-term suppression of the innate immune system function (Burrell *et al.*, 1996; Raidal *et al.*, 2000; Robson *et al.*, 2003), followed by an increase of infections in horses during training (Burrell *et al.*, 1996). Most isolated bacterial species are airway epithelia inhabitants, which under stress conditions and reduced immunity of the body, can cause serious illness. The differences between the two groups are in percentages and could be explained by the wider movement of horses from the semi-intensive raising system.

#### 4. Conclusion

The results indicated that, in spite of the sitting period, the workout background not only influenced the carried microflora but also the cell-mediated response to plant extract stimulation, thus potential immune stimulating and antibacterial treatments should be designed based on workout and also the productive period.

#### Conflict of interest

We declare that we have no conflict of interest.

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