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Assessment of probiotic effects of "OmniBiotic Cat & Dog" on primary isolated equine immune cells

Evaluierung der probiotischen Wirkung von „Omnibiotic Cat & Dog" auf primär isolierte Immunzellen von Pferden

Diploma thesis

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Submitted by

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1. Abstract

English

The product „OmniBiotic" from ALLERGOSAN is a probiotic that is used in both human and veterinary medicine to help rebuild the physiological intestinal flora. The product used in this work, "OmniBiotic Cat & Dog", contains two bacterial strains: *Enterococcus faecium DSM10663/NCIMB* and *Lactobacillus acidophilus CECT 4529*, whose possible positive immunomodulatory influence on equine peripheral blood mononuclear cells was investigated in this diploma work. Therefore, the cell behavior regarding the growth, proliferation and/or death of the PBMCs was monitored and evaluated using the "Incucyte S3" and the "Countess III". In addition, an antibody staining of various immune cells such as CD4, CD8, Mono-APC and IL-17 was carried out to detect T-cell subsets using immunofluorescence in the "Incucyte S3" and the "BD Canto" flow cytometer.

The results of this study provided evidence that the probiotic product can indeed have an immunomodulatory effect on equine PBMCs when cultured with certain medium and serum substitute. In detail, it was found, that the equine PBMCs survived better the RPMI medium in comparison to the DMEM medium and seem to be most comfortable in fetal bovine serum compared to individual horse serum and FC1 serum, even though there may be individual differences and deviations.

German

Das Probiotikum „OmniBiotic“ der Firma ALLERGOSAN ist ein Produkt, das sowohl in der Human- als auch der Veterinärmedizin eingesetzt wird, um unter anderem dem Wiederaufbau der physiologischen Darmflora zu helfen. Es gibt mittlerweile verschiedene Produkte, die unterschiedlich viele Bakterienstämmen enthalten. Das in dieser Arbeit verwendete Produkt „OmniBiotic Cat & Dog“ enthält zwei Bakterienstämme: *Enterococcus faecium DSM10663/NCIMB* and *Lactobacillus acidophilus CECT 4529*, deren möglicher positiver immunmodulatorischer Einfluss auf equine periphere mononukleare Blutzellen getestet wurde. Dafür wurde das Zellverhalten hinsichtlich des Wachstums, der Proliferation und/ oder Versterben der PBMCs mit dem „Incucyte S3“ und der „Countess III“ überwacht und ausgewertet. Darüber hinaus wurde noch eine Antikörperfärbung verschiedener Immunzellen wie CD4, CD8, Mono-APC und IL-17 durchgeführt, um diese T-Zell Untergruppen erfolgreich mittels Immunfluoreszenz im „Incucyte S3“ sowie im Durchflusszytometer „BD-Canto“ nachzuweisen.

Die Ergebnisse der Studie konnten den Nachweis erbringen, dass das Probiotikum in der Tat einen positiven immunmodulatorischen Effekt auf die PBMCs von Pferden haben kann, wenn sie mit bestimmten Medien und Serumzusätzen kultiviert werden. Im Einzelnen wurde festgestellt, dass die equinen PBMCs im RPMI-Medium eine größere Überlebensrate zeigten als im DMEM-Medium und sich im fetalen Rinderserum, im Vergleich zu den individuellen Pferdeseren und dem FC1-Serum, am wohlsten fühlten, auch wenn es dabei individuelle Unterschiede und Abweichungen gab.

2. List of abbreviations

PBMCs = peripheral blood mononuclear cells

EDTA = Ethylenediaminetetraacetic

RT = room temperature

ACC = acceleration

DEC = declaration

sec = seconds

min = minutes

rpm = runs per minute

g = gravity

cfu = colony forming units

°C = degree celsius

PBS = phosphate buffered saline

BSA = bovine serum albumin

FBS = fetal bovine serum

HS = horse serum

FC1 = fetal clone serum 1

ConA = ConcavalinA

OB = OmniBiotic Cat & Dog

RPMI = Roswell Park memorial institute medium

DMEM = Dulbecco's modified eagle medium

3. Introduction

The role of horses and the human-horse relationship has changed considerably during domestication. At the beginning of the human-horse relationship, the horse served humans primarily as a food-providing animal, but its role as a means of transport grew until it finally became a companion animal in its own right (1). Due to this development of the horse's position in society and the human-horse relationship, it is hardly surprising that in the meantime the equine medicine has also reached a very high level and that research is also far advanced. Thus, the current state of knowledge about the health of horses as well as the diagnostic and therapeutic possibilities are almost comparable to those of the human medicine.

Immune system

'The immune system is a network of cells and proteins that interact in tissues and organs to protect the body from infection, and also to promote healing' (2) – this statement can be applied to both humane and equine medicine, which will be discussed in more detail below. The equine immune system consists of several components and can be divided into two different categories: the innate immunity and the adaptive immune system (3). The innate immune system is the first defense line and, in addition to immune cells like neutrophiles, macrophages, dendritic cells, mast cells and natural killer cells, it also includes physical barriers such as the skin, epithelia and mucosa and is thus able to ward off an initial threat of infection by initializing an inflammatory reaction, even if only for a short time (3). In contrast, the adaptive immune system is slower, but more specific and potent, which is primarily due to the two main lymphoid cells: the antigen specific T-& B- lymphocytes (3). B-cells mature in the bone marrow and later differentiate in the lymphatic tissue under the influence of pathogenic antigens, which are presented to the B-cells by dendritic cells (2). This antigen contact and the interaction with T-cells lead to a clonal expansion of the B-cells: either the B-cells proliferate directly into plasma cells and release the immunoglobulin M or the B-cells adapt their receptors individually to the antigen so that more specific immunoglobulins can be formed and thus antibodies are released and an immunological memory is formed (2). T- cells also mature in the bone marrow but, unlike the B-cells, they differentiate in the thymus by forming T-cell receptors (2). In horses several T-cell receptor types are known: CD3, CD4, CD8 (2). Those T-cells with their

individual receptors circulate in the blood and tissue and can trigger an immune response through the production and release of cytokines, such as interleukin-17 (IL-17) (2). T-cells become activated, similar to the B-cells, by dendritic cells, which thus represent the link between the innate and adaptive immune system, by migrating with the antigen from the tissue into the regional lymph nodes and presenting the antigen to the CD4 helper T-cells as well as to the B-cells (2).

B-& T-cells as well as monocytes and natural killer cells belong to the peripheral blood mononuclear cells (PBMCs) (4). PBMCs can be isolated from the peripheral blood and contain all blood cells with a round nucleus (4). Studies in human medicine have shown, that PBMCs are well-suited for the investigation of immune modulatory effects of food bioactive compounds (4), which is why PBMCs are also used in the experiment described in this trial, to detect the assessment of probiotic effects on primary isolated equine immune cells.

Probiotics

Studies showed, that probiotics as a feed additive can have a positive effect on the immune system due to their metabolites, their cell wall components and DNA (5).

Probiotics in general belong to the living microorganisms that could have positive effects on the host if they are consumed in the correct amount (6). The World Health Organization defines probiotics as *'life microorganisms, that when administered orally at adequate concentrations, provide a beneficial effect beyond that of their nutritional value'* (5).

Probiotics can for example support the health of horses with gastrointestinal problems, reduce the risk of gastrointestinal diseases or even positively influence diseases independent of the gastrointestinal tract (6). They achieve their positive effect by influencing the microbiome in several ways. The main discussed mechanisms of action are: production of antimicrobial compounds that target intestinal pathogens, general stimulation of the immune system and resistance to colonization (5).

Although probiotics are widely available today, comparatively cheap and have few side effects, there is only little scientific evidence for commercial probiotic formulations in horses (5). However, some promising results have already been achieved *in vitro*, although proving the positive effects *in vivo* is much more difficult (5). Due to the scarcity of research results so far

and the difficulty of positive *in vivo* proof, the trial described in this thesis refers to the influence of probiotics on the immune system, especially PBMCs of horses *in vitro*.

Despite a few studies, there are nevertheless already some findings on the possible use of probiotics in horses: for example, V. Urubschurov *et al.* tested the influence of two bacterial strains (*Lactobacillus rhamnosus* and *Enterococcus faecium*) on the fecal microbiome of foals (7), as did Ch. Ströbel *et al.* who studied the influence of the same two bacterial strains in neonatal diarrhea in foals (8). In addition, the study by A. Zeyner presents the possibility of probiotics to prevent digestive problems in horses and to positively support their fermentation in the large intestine (9).

The effects of probiotics have not been studied in detail in horses yet, but in other animal models detailed modes of action and effects could already be found; the review by S. Patel *et al.* shows the connection between probiotics and the innate immune system in various animals, based on in vitro experiments on fish, poultry, mice, pigs, monkeys and humans (10). The review also discusses the different ways and targets of probiotics and their effects on the immune system and how they can influence the immune system through effector molecules, control of intestinal mucus production and bacterial restructuring of the intestinal flora (10).

Although there are more specific studies e.g. in dogs and pigs where immunomodulatory effects caused by the bacterial strain *Enterococcus faecium* have been observed (11,12). The study by N. Bruni *et al.* showed that not only the immune system of dogs but also their general behavior as well as the fecal parameters, nutrition and the gastrointestinal microbiome can be positively influenced, in this experiment mainly by the probiotic bacterial strain of *Lactobacillus acidophilus* (13). S. P. Marelli *et al.* came to the same conclusions in his study (14). With regard to the probiotic bacterium *Enterococcus faecium*, J. Benyacoub *et al.* concluded, that oral substitution can increase immune function in the long term by influencing the immune system via the intestinal lymph follicles (15). S. Alonge *et al.* also describes in his study that the oral substitution of probiotics not only influences the immune system of the treated animal, but even has positive effects on the colostrum quality and can thus improve the supply of immunoglobulins to the puppies (16).

However, not only in veterinary medicine, but also in human medicine, research has been conducted on the influence of the two probiotic bacterial strains, especially in connection with the immune system; in the study from Molina MA *et al.* he came to the conclusion that

Enterococcus faecialis activates dendritic cells of the immune system, which in turn increased the expression of cells of the adaptive immune system (17). The review by L. Santiago-López *et al.* also describes that the probiotic strain of *Lactobacillus* indeed influences the immune system in humans as well as in several animals by being recognized by the macrophages of the intestine, taken up and transported to the deeper lymphoid follicles, which in turn react by stimulating cytokines and immunoglobulins, thus influencing the immune system (18). Also N. Fumiko *et al.* found a positive correlation between the oral intake of Lactobacilli and reduced levels of natural killer cells in the peripheral blood of humans (19).

Currently, there are a few probiotic products approved for horses on the market (5). In this pilot study the probiotic product "OmniBiotic Cat & Dog", developed for the usage in cats and dogs by the institute ALLERGOSAN, with its two bacterial strains *Enterococcus faecium DSM10663/NCIMB* and *Lactobacillus acidophilus CECT 4529* (20) is selected for investigation on equine PBMCs. . The product is used as an oral feed additive to stabilize the intestinal flora (20). In addition to the already mentioned two strains of bacteria, the product also contains maize starch, mannan-oligosaccharides as a high quality dietary fiber and maltodextrin and is commercially available as a powder with 1,25 billion cfu per portion (20).

Media and serum substitutes

Moreover, this trial includes two further experiments: firstly, to test in which medium-serum combination the equine PBMCs are more vital. For this purpose, Roswell Park Memorial Institute (RPMI) and Dulbecco's Modified Eagle Medium (DMEM) were used. Secondly, the supplementation of individual serum of the horses, fetal bovine serum (FBS) or Fetal Clone 1, an artificial medium supplement was tested. The reason for this question is, among other things, the attempt to establish serum alternatives in addition to the FBS medium, as its quality and reproducibility have many disadvantages, especially with regard to animal welfare (21). The horse serum used for this experiment comes individually from each horse and can be obtained at the same time as the PBMCs are isolated from the blood. At last, Concanavalin A (ConA) is used as a positive control compared to the negative control without any treatment and the added probiotic product "OmniBiotic Cat & Dog". ConA is a lectin which is mitogenic for

lymphocytes, reduces the mobility of immunoglobulin receptors on the lymphoid cell surface and suppresses the phagocytosis through polynuclear leucocytes (22).

Visualization and monitoring

In order to monitor and document the growth behavior of the equine PBMCs visually regarding their cell number, the “Incucyte S3” is used as a live cell imaging device. The “Incucyte S3” makes it possible to incubate the cells *in vitro* in physiological conditions and to document their vitality and behavior as well as cell to cell interactions around the clock without disturbing the cells or having to remove them from their physiological environment (23). Not only the cell numbers can be measured in individually adjustable time intervals over the desired period, but also a visual documentation of the behavior of the cells in form of pictures and/or videos is possible (23). To have a comparison regarding the visualization of the cells, they can also be displayed in a flow cytometry. This analysis tool allows large cell populations to be analyzed and visualized down to their smallest cell unit (24). As a light source, lasers are most commonly used in the flow cytometry to produce scattered as well as fluorescent signals which are captured by detectors (25). Most commonly stained monoclonal antibodies are used in the flow cytometry, as these show many different spectra when coupled with different fluorochromes and are easy to evaluate with markers (24). For this reason, the method of flow cytometry is used in this experiment to have a comparison to the visual representation of the stained antibodies from the “Incucyte S3”.

4. Hypothesis

The probiotic product „OmniBiotic Cat & Dog" has direct immunomodulatory effects to the adaptive immune cells (PBMCs) in equine blood. Based on this hypothesis, higher cell proliferation rates are expected within the cells which were treated with the probiotic product "OmniBiotic Cat & Dog".

Furthermore, the PBMCs react different in terms of their growth, survival, and proliferation on different serum substitutes (horse serum vs fetal bovine serum vs fetal clone 1 serum substitute) as well as with different basal mediums (RPMI vs DMEM). The cell vitality is expected to be higher with the horse serum, as equine PBMCs are more comfortable in this medium supplement because it is their natural reservoir.

Immune phenotyping of adaptive immune cells utilizing commercially available antibodies such as CD4, CD3, CD8, Monocytes, B-cells and Interleukin 17 can be visualized with the flow cytometer "BD Canto" as well as with the live cell imaging device "Incucyte S3".

5. Material and methods

Devices

- Centrifuge Mikro Star 17R
- Centrifuge Mega Star 1.6R
- Laminar Flow Hood MSC Advantage
- Steel bath, Precision GP 10
- Incubator, HeraCell VIOS 160i
- Cell Counter „Countess III FL"
- Incucyte S3 Live-Cell Analysis Instrument

Materials

- Pipette 2-20µl
- Pipette 20-200µl
- Pipette 100-1000µl
- Pipetboy, Levo ME
- DNAse/ RNAse free sterile filter tips (10µl, 20µl, 200µl, 1250µl)
- Serological pipet (5ml, 10ml, 25ml)
- DNase/ RNase free sterile reaction tubes (1,5ml)
- Centrifuge tube (15ml, 50ml)
- Cell culture flask, Nunc EasY Flask, 100cm^2
- Cell Counting chambers, Countess TM
- Ice
- Timer
- Rack for 2ml, 15ml, 50ml tubes
- Paper towel
- Marker for labelling
- Protection gloves

Solutions, Chemicals, Reagents, Kits

- EDTA-Blood from horses
- Histopaque -1077, 100ml, sterile filtered, densitiy:1.077g/mol
- Phosphat buffered saline, 500ml, DPBS (1X) without CaCl2/MgCl2
- 1X PBS/ BSA (1%)

- Trypan blue staining
- red blood cell lysis buffer
- Cell cultivation medium, 500ml, RPMI Medium 1640 (1X) with L-Glutamine
- Cell cultivation medium, 500ml, DMEM Medium
- Fetal bovine Serum FBS
- Concanavalin A (ConA) Solution 500X
- OmniBiotic Cat & Dog
- Anti-CD4 antibody (FITC)
- Anti-CD8 antibody (PE)
- Anti-CD3 antibody (Pacific Blue)
- Anti-B-Cells antibody (PE)
- Anti-Macrophage/ Monocyte antibody (APC)
- Anti-Interleukin 17A antibody (FITC)
- Water

Preparation of blood samples

The trials described below were conducted on three different time periods with two to three horses per trial. The approach/ procedure was the same for every run and the trial procedure is therefore described collectively from here on. Slight deviations are specifically broken down individually for the respective sample.

(Outside the laminar flow)

Blood samples are taken from seven different horses into EDTA-Vacutainers and immediately stored on ice.

- Horse 1 in 3 EDTA-Vacutainers á 3ml
- Horse 2 in 3 EDTA-Vacutainers á 3ml
- Horse 3 in 3 EDTA-Vacutainers á 3ml
- Horse 4 in 4 EDTA-Vacutainers á 3ml
- Horse 5 in 4 EDTA-Vacutainers á 3ml
- Horse 6 in 3EDTA-Vacutainers á 9ml
- Horse 7 in 3EDTA-Vacutainers á 9ml

Seven 50ml tubes are prepared and labeled from one to seven for each blood sample. The blood from all EDTA-Vacutainers per horse are gently transferred with a 5ml pipet into the 50ml tubes without any air bubbles. Each of the now filled 50ml centrifuge tubes has a volume of 10-25ml blood inside of it.

- Horse 1 10ml
- Horse 2 10ml
- Horse 3 10ml
- Horse 4 15ml
- Horse 5 15ml
- Horse 6 25ml
- Horse 7 25ml

After the blood is transferred the EDTA-Vacutainers are getting washed out with PBS (RT) which also is transferred to the 50ml tubes. Every tube now gets filled up with the PBS to dilute the blood samples in ratio 1:1.

- Horse 1 = 10ml Blood + 10ml PBS (RT)
- Horse 2 = 10ml Blood + 10ml PBS (RT)
- Horse 3 = 10ml Blood + 10ml PBS (RT)
- Horse 4 = 15ml Blood + 15ml PBS (RT)
- Horse 5 = 15ml Blood + 15ml PBS (RT)
- Horse 6 = 25ml blood + 25ml PBS (RT)
- Horse 7 = 25ml blood + 25ml PBS (RT)

Seven new 50ml tubes get prepared and labeled from 1-7 for each blood sample and loaded with Histopaque. Now the diluted blood samples are loaded in ratio 2:1 with an 25ml pipet on the prepared Histopaque in the 50ml tubes. For that step, the 50ml tubes with the Histopaque inside must be held nearly transversally and the diluted blood samples need to rinse very carefully and slowly along the wall of the tube down to the Histopaquelayer, because the blood samples and the Histopaque are not allowed to mix up!

Due to the amount of blood in horses 6 and 7, their blood is divided into two tubes as otherwise there would be problems with filling up the tubes.

- Horse 1 = 20ml diluted blood on 9ml Histopaque
- Horse 2 = 20ml diluted blood on 10ml Histopaque
- Horse 3 = 20ml diluted blood on 10ml Histopaque
- Horse 4 = 30ml diluted blood on 15ml Histopaque
- Horse 5 = 30ml diluted blood on 15ml Histopaque
- Horse 6.1 = 25ml diluted blood on 12,5ml Histopaque
  Horse 6.2 = 25ml diluted blood on 12,5ml Histopaque
- Horse 7.1 = 25ml diluted blood on 12,5ml Histopaque
  Horse 7.2 = 25ml diluted blood on 12,5ml Histopaque

After the transfer onto the Histopaque the samples get centrifuged for 25min at 20° at 2000rpm with acceleration (ACC) at 7 and brake (DEC) at 4.

The next steps were carried out inside a laminar flow to ensure as least as possible contamination for the cultivation.
During centrifugation a fresh 15ml tube is prepared and labeled for each sample.
The centrifuged samples are placed on a rack in the laminar flow.
The centrifuged samples show three different layers: the first layer on the surface represents the serum, the second cloudy layer in the middle is the buffy coat which implies the PBMCs and the third layer at the bottom includes the red blood cells (Figure 1).

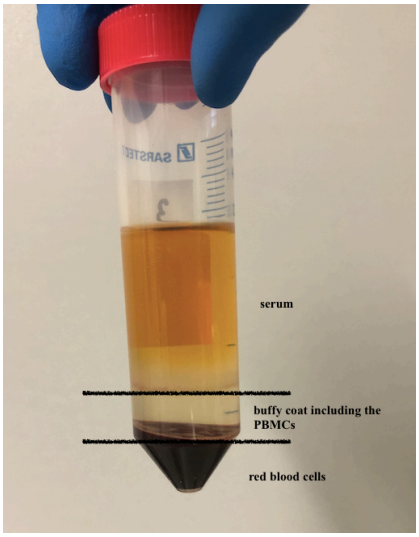

Figure 1: *centrifuge blood samples*
the serum is at the top in a slightly yellowish color, the buffy coat with the required PBMCs is in the middle and the red blood cells are at the bottom.

The serum at the upper layer of the centrifuged samples is removed carefully with an 25ml pipet. The serum of the samples 1, 2, 3 get stored together in a fresh 50ml tube at 37°C in a steal bath and will be used later. The serum of the samples 4 and 5 as well as of the samples 6 and 7 also get stored together in a fresh 50ml tube at 37°C in a steal bath and will be used later. The cloudy middle layer of the centrifuged samples with the buffy coat contains the desired PBMCs including the white blood cells and is carefully pipetted with an 1000µl pipet without swirling up the underlying layer of red blood cells. The buffy coat of each sample is transferred to the already prepared and labeled fresh 15ml tubes. Since the amount of blood was so large in horse 6 and 7 and these were already divided into 2 portions per horse as described above, this now divides into samples 6.1, 6.2, 7.1 and 7.2 created in this way is also retains here and the buffy coat of the respective samples is pipetted into individual 15ml tubes.
The red blood cells can be thrown away for this approach.
The 15ml tubes with the buffy coat inside are filled up to a total volume of 12ml with PBS. After adding the PBS, the tubes get closed and inverted for several times to mix the buffy coat with the added PBS. Afterwards, all samples are centrifuged at 1200rpm for 8min at room temperature. ACC and DEC are both set at 7.
After centrifugation the samples get back on a rack and placed inside the laminar flow again. At the bottom of each sample a pellet has settled. The supernatant above the pellet can be removed and discarded with a 5ml pipet.
Depending on the size and color of the pellet it needs to get erythrolized if it is too red because too many red blood cells are remained. Depending on the amount of redness 1-3ml of red blood cell lysis buffer get added to the pellet and resuspended carefully. Afterwards the tube is stored on ice for 5min. By adding PBS to the tube up to a total volume of 14ml the red blood cell lysis is stopped. The sample gets resuspended, closed, inverted, and centrifuged for 10min at 4°C and 1200rpm. If the color of the pellet is still too red after the Erythrolysis, the step needs to be repeated until the sample is sufficiently erythrolyzed.

- horse 1: erythrolized 3 times
  first time with 3ml erythrolysis buffer
  second time with 2ml erythrolysis buffer (the pellet was still a bit red)
- horse 1*: erythrolized one more time with 2ml erythrolysis buffer

- horse 2: erythrolized 2 times
  first time with 3ml erythrolysis buffer
  second time with 2ml erythrolysis buffer (then the pellet looked fine and not red anymore)
- horse 3: no erythrolysis needed
- horse 4: no erythrolysis needed
- horse 5: erythrolized one time with 3ml erythrolysis buffer (the pellet was still a bit red)
- horse 6.1: erythrolized one time with 2ml erythrolysis buffer
- horse 6.2: erythrolized one time with 2ml erythrolysis buffer
- horse 7.1: erythrolized 2 times > both times with 3ml erythrolysis buffer
- horse 7.2: erythrolized 2 times > both times with 3ml erythrolysis buffer

If the pellets don't look too red anymore, 1ml of 1% PBS/BSA can be added and resuspended to the pellet. 20µl of the resuspended cells get transferred to a fresh and labeled 1,5ml tube for the cell counting. The 15ml tube with the resuspended cells gets filled up to a total volume of 10ml with RTU media to make the cells feel more comfortable.

Cell counting

Cell counting was performed by Countess III (ThermoFisher) which is an image based automated cell counting device. In the 1,5ml tube with 20µl of the resuspended cells inside, 20µl Trypan blue is added with a pipet and then resuspended. Then 10µl of the sample are transferred into a chamber of a counting slide. The counting slide get inserted in the cell counter and the cell counting get started. Notes are taken of the total number of cells, the absolute and percentual living and dead cells as well as the cell size of the living and dead cells.
The counting process was repeated with the other samples.
Each sample should be counted at least two times.

Preparation of cell culture medium

The collected serum samples get filtered through a syringe pre-filter and are stored in a 50ml tube (one per serum sample) in a steal bath at 37°C.

- 50ml tube with the serum from the horses 1, 2, 3
- 50ml tube with the serum from the horses 4, 5
- 50ml tube with the serum from horse 6
- 50ml tube with the serum from horse 7

The FBS and the FC1 is also heated up in the steal bath at 37°C.

Then the mediums with RPMI or DMEM and FBS as well as the HS and the FC1 as a serum substitute are made:

- Samples 1, 2, 3
    - 37,5ml RPMI medium + 3,75ml FBS = 10% solution in a 50ml tube
        - 4 x 15ml tubes labeled with 10% FBS medium – per tube 3ml medium
            - 1 tube for horse 1 = 12,5µl cells in 3ml
            - 1 tube for horse 1* = 41,2µl in 3ml
            - 1 tube for horse 2 = 52,8µl in 3ml
            - 1 tube for horse 3 = 500µl in 3ml
    - 37,5ml RMPI medium + 3,75ml HS = 10% solution in a 50ml tube
        - 4 x 15ml tubes labeled with 10% HS medium – per tube 3ml medium
            - 1 tube for horse 1 = 12,5µl cells in 3ml
            - 1 tube for horse 1* = 41,2µl in 3ml
            - 1 tube for horse 2 = 52,8µl in 3ml
            - 1 tube for horse 3 = 500µl in 3ml
- Samples 4 and 5
    - 25ml RPMI medium + 2,5ml FBS = 10% solution in a 50ml tube
        - 1 x 15ml tube labeled with 10% FBS medium = 3ml medium + 500µl cells from horse 4
        - 1x 50ml tube labeled with 10% FBS medium = 18ml medium + 201µl cells from horse 5

- 25ml RPMI medium + 2,5ml HS = 10% solution in a 50ml tube
  - 1 x 15ml tube labeled with 10% HS medium = 3ml medium + 500µl cells from horse 4
  - 1x 50ml tube labeled with 10% HS medium = 18ml medium + 201µl cells from horse 5

- Samples 6 and 7
  - 30ml RPMI medium + 3ml FBS = 10% solution in a 50ml tube
  - 20 ml RPMI medium + 2ml HS = 10% solution in a 50ml tube
  - 15ml RPMI medium + 1,5ml FC1 = 10% solution in a 50ml tube
  - 15ml DMEM medium + 1,5ml HS = 10% solution in a 50ml tube

Preparation of the antibody stainings

For the antibody staining 5x10^5 cells per well are required which must have been previously treated with 1% PBS/ BSA to control the unspecific binding to the FC receptor. The cells were pelletized for the staining and are therefore centrifuged at 800g 10min at RT. After the centrifugation the supernatant gets removed and discarded. The amount of cells required is determined from the cell count.

- Horse 6 = Cells of the horses 6.1 und 6.2 are mixed in a new 50ml tube and then have a mean number of living cells of 8,77x10^6 cells/ml
- Horse 7 = Cells of the horses 7.1 und 7.2 are mixed in a new 50ml tube and then have a mean number of living cells of 1,94x10^7 cells/ml

Per horse six antibodies should be tested. That is why six 1,5ml tubes per horse are needed. The tubes are labeled and filled with the amount of cell pellet calculated. After the cell pellets have been prepared, they are mixed up with 50µl PBS and the respective antibodies:

- Horse 6
  - CD4-Fitc = 17,54µl cell pellet + 50µl PBS + 1µl antibody
  - CD8-PE = 17,54µl cell pellet + 50µl PBS + 1µl antibody
  - CD3-PacBlue = 17,54µl cell pellet + 50µl PBS + 1µl antibody
  - Monocytes-APC = 17,54µl cell pellet + 50µl PBS + 5µl antibody
  - B-cells-PE = 17,54µl cell pellet + 50µl PBS + 5µl antibody
  - Interleukin-17-FITC = 17,54µl cell pellet + 50µl PBS + 5µl antibody

- Horse 7
    - CD4-Fitc = 38,8µl cell pellet + 50µl PBS + 1µl antibody
    - CD8-PE = 38,8µl cell pellet + 50µl PBS + 1µl antibody
    - CD3-PacBlue = 38,8µl cell pellet + 50µl PBS + 1µl antibody
    - Monocytes-APC = 38,8µl cell pellet + 50µl PBS + 5µl antibody
    - B-cells-PE = 38,8µl cell pellet + 50µl PBS + 5µl antibody
    - Interleukin-17-FITC = 38,8µl cell pellet + 50µl PBS + 5µl antibody

After that the cells with the antibodies need to incubate on ice in the dark for at least 30min but no longer than 1h. Then the mixes are extinguished/ washed off by adding 1ml of 1% PBS/ BSA and centrifugation at 800g 10min at 10°C. The supernatant again gets lifted off and the pellets, which are located on the ground of the tube, with the cells and the antibodies are resuspended in 50µl PBS. Now the wells on the 24-well plate can be assembled.

Preparation of the probiotic product "OmniBiotic Cat & Dog"

One forth from the provided spoon of the "OmniBiotic Cat & Dog" contains 75g and thus 1,25x10^9 colony forming units of the bacterial strains *Enterococcus faecium DSM10663/NCIMB* and *Lactobacillus acidophilus CECT 4529*. Those 75g are dissolved in 100ml Water and well mixed. Then 1ml of the dissolved solution is transferred into a 1,5ml collecting tube which now contains 1,25x10^7 colony forming units.

From this 1,5ml collecting tube 20µl with 2,5x10^5 cfu are placed onto the wells with the OB for the horses 1, 1*, 2, 3.

For the horses 4 and 5 only 2x10^5 cfu per well are needed which is why only 16µl are placed on the wells with the OB.

For the horses 6 and 7 a different amount of cfu per well are needed for the different plates

- Plate 4: 2x10^5 cfu per well = 16µl
- Plate 6: 5x10^5 cfu per well = 40µl
- Plate 7: 1x10^6 cfu per well = 80µl

Make sure to vortex the 1,5ml tube with the OmniBiotic Cat & Dog solution before assembling the wells so that the hole solution is mixed up well.

Preparation of the cell culture plates

The cell culture plates have 24 wells and are filled according to the following pipette schemes:

Horse 1 FBS CONTROL	Horse 1 FBS CONA	Horse 1 FBS OB	Horse 1 HS CONTROL	Horse 1 HS CONA	Horse 1 HS OB
Horse 1* FBS CONTROL	Horse 1* FBS CONA	Horse 1* FBS OB	Horse 1* HS CONTROL	Horse 1* HS CONA	Horse 1* HS OB
Horse 2 FBS CONTROL	Horse 2 FBS CONA	Horse 2 FBS OB	Horse 2 HS CONTROL	Horse 2 HS CONA	Horse 2 HS OB
Horse 3 FBS CONTROL	Horse 3 FBS CONA	Horse 3 FBS OB	Horse 3 HS CONTROL	Horse 3 HS CONA	Horse 3 HS OB

Table 1: *plate 1 - pipet scheme for the horses 1, 1*, 2, 3 (for the Incucyte)*
*> 2,5*10^5 cells/ well (in all wells)*
*> 2,5*10^5 cfu "OmniBiotic Cat & Dog" per well (labeled wells with "OB")*
> 2µl ConA/ Well (labeled wells with "ConA")

horse 4 FBS CONTROL	Horse 4 FBS CONA	Horse 4 FBS OB	Horse 4 HS CONTROL	Horse 4 HS CONA	Horse 4 HS OB
Horse 5 FBS CONTROL	Horse 5 FBS CONA	Horse 5 FBS OB	Horse 5 HS CONTROL	Horse 5 HS CONA	Horse 5 HS OB
Horse 5 FBS CONTROL	Horse 5 FBS CONA	Horse 5 FBS OB	Horse 5 HS CONTROL	Horse 5 HS CONA	Horse 5 HS OB

Table 2: *plate 2 - pipet scheme for the horses 4 and 5 (for the Incucyte)*
*> 2*10^5 cells/ well (in all wells)*
*> 2*10^5 cfu "OmniBiotic Cat & Dog" per well (labeled wells with "OB")*
> 2µl ConA/ Well (labeled wells with "ConA")

Horse 5 FBS CONTROL	Horse 5 FBS CONA	Horse 5 FBS OB	Horse 5 HS CONTROL	Horse 5 HS CONA	Horse 5 HS OB
Horse 5 FBS CONTROL	Horse 5 FBS CONA	Horse 5 FBS OB	Horse 5 HS CONTROL	Horse 5 HS CONA	Horse 5 HS OB
Horse 5 FBS CONTROL	Horse 5 FBS CONA	Horse 5 FBS OB	Horse 5 HS CONTROL	Horse 5 HS CONA	Horse 5 HS OB
Horse 5 FBS CONTROL	Horse 5 FBS CONA	Horse 5 FBS OB	Horse 5 HS CONTROL	Horse 5 HS CONA	Horse 5 HS OB

Table 3: *plate 3 - pipet scheme for the horse 5 (for the incubator for cell harvesting after 24h and 48h)*

*> 2*10^5 cells/ well (in all wells)*
*> 2*10^5 cfu "OmniBiotic Cat & Dog" per well (labeled wells with "OB")*
> 2µl ConA/ Well (labeled wells with "ConA")

Per well 1ml of FBS or HS medium with the proportional cell count is filled in the wells.

- Horses 1, 1*, 2, 3 = 2,5*10^5 cells per well
- Horses 4 and 5 = 2*10^5 cells per well

Per well 2µl of ConA is added in the labeled wells.

Per well of the solution from "OmniBiotic Cat & Dog" is added in the labeled wells

- horses 1, 1*, 2, 3 = 2,5*10^5 cfu per well = 20µl
- horses 4 and 5 = 2x10^5 cfu per Well = 16µl

Horse 6 RPMI+FBS	Horse 6 RPMI+FBS ConA	Horse 6 RPMI+FBS OB	Horse 7 RPMI+FBS	Horse 7 RPMI+FBS ConA	Horse 7 RPMI+FBS OB
Horse 6 RPMI+HS	Horse 6 RPMI+HS ConA	Horse 6 RPMI+HS OB	Horse 7 RPMI+HS	Horse 7 RPMI+HS ConA	Horse 7 RPMI+HS OB
Horse 6 RPMI+FC1	Horse 6 RPMI+FC1 ConA	Horse 6 RPMI+FC1 OB	Horse 7 RPMI+FC1	Horse 7 RPMI+FC1 ConA	Horse 7 RPMI+FC1 OB
Horse 6 DMEM+HS	Horse 6 DMEM+HS ConA	Horse 6 DMEM+HS OB	Horse 7 DMEM+HS	Horse 7 DMEM+HS ConA	Horse 7 DMEM+HS OB

Table 4: *plate 4 - pipet scheme for the horses 6 and 7 (for the Incucyte)*
> 1ml medium/ per well (in all wells)
*> 2*10^5 cells/ well (in all wells) = 24,72µl per well for horse 6 and 7,57µl per well for horse 7*
*> 2*10^5 cfu "OmniBiotic Cat & Dog" per well (labeled wells with "OB") = 16µl*
> 2µl ConA/ Well (labeled wells with "ConA")

Horse 6 RPMI+FBS CD4-Fitc	Horse 6 RPMI+FBS B-cells-PE		Horse 7 RPMI+FBS CD4-Fitc	Horse 7 RPMI+FBS B-cells-PE	
Horse 6 RPMI+FBS CD8-Pe	Horse 6 RPMI+FBS IL-17-FITC		Horse 7 RPMI+FBS CD8-Pe	Horse 7 RPMI+FBS IL-17-FITC	
Horse 6 RPMI+FBS CD3-PacBlue			Horse 7 RPMI+FBS CD3-PacBlue		
Horse 6 RPMI+FBS Mono-APC			Horse 7 RPMI+FBS Mono-APC		

Table 5: *plate 5 - pipet scheme for the horses 6 and 7 (for the Incucyte)*
> 1ml medium/ well (in all wells)
*> 5*10^5 cell pellet + respective antibody/ well (in all wells) = 24,72µl per well for horse 6 and 7,57µl per well for horse 7*

Horse 6 RPMI+FBS	Horse 6 RPMI+FBS ConA	Horse 6 RPMI+FBS OB	Horse 7 RPMI+FBS	Horse 7 RPMI+FBS ConA	Horse 7 RPMI+FBS OB
Horse 6 RPMI+HS	Horse 6 RPMI+HS ConA	Horse 6 RPMI+HS OB	Horse 7 RPMI+HS	Horse 7 RPMI+HS ConA	Horse 7 RPMI+HS OB
			Horse 7 RPMI+FC1	Horse 7 RPMI+FC1 ConA	Horse 7 RPMI+FC1 OB
			Horse 7 DMEM+HS	Horse 7 DMEM+HS ConA	Horse 7 DMEM+HS OB

Table 6: *plate 6 - pipet scheme for the horses 6 and 7 (for the incubator for cell harvesting after 24)*
> 1ml medium/ well (in all wells)
*> 5*10^5 cells/ well (in all wells) = 61,8µl per well for horse 6 and 18,94µl per well for horse 7*
*> 5*10^5 cfu "OmniBiotic Cat & Dos" per well (labeled wells with "OB") = 40µl*
> 2µl ConA/ Well (labeled wells with "ConA")

			Horse 7 RPMI+FBS	Horse 7 RPMI+FBS ConA	Horse 7 RPMI+FBS OB
			Horse 7 RPMI+HS	Horse 7 RPMI+HS ConA	Horse 7 RPMI+HS OB
			Horse 7 RPMI+FC1	Horse 7 RPMI+FC1 ConA	Horse 7 RPMI+FC1 OB
			Horse 7 DMEM+HS	Horse 7 DMEM+HS ConA	Horse 7 DMEM+HS OB

Table 7: *plate 7 - pipet scheme for horse 7 (for the incubator for cell harvesting after 24h and 48h) antibody staining and counting in the flow cytometer*
> 1ml medium/ well (in all wells)
> 1x10^6 cells/ well (in all wells) = 37,88µl per well for horse 7
> 1x10^6 cfu "OmniBiotic Cat & Dog" per well (labeled wells with "OB") = 80µl
> 2µl ConA/ Well (labeled wells with "ConA")

Per well 1ml of RPMI or DMEM medium and FBS, HS or FC1 medium is placed in the wells.

- Plate 4 = 2*10^5 cells per well
- Plate 5 = 2*10^5 cells per well
- Plate 6 = 5*10^5 cells per well
- Plate 7 = 1*10^6 cells per well

Per well the required amount of cells or their pellet and the antibodies are filled in the wells.

Per well 2µl of ConA is added in the labeled wells.

Per well the required amount of the solution from "OmniBiotic Cat & Dog" is added in the labeled wells:

- Plate 4 = 2*10^5 cfu per well = 16µl
- Plate 6 = 5*10^5 cfu per well = 40µl
- Plate 7 = 1*10^6 cfu per well = 80µl

Cultivation of PBMCs

The first and second cell culture plates with the horses 1, 1*, 2, 3 as well as with the horses 4 and 5 are placed in the “Incucyte S3” for 4 days and every 4 hours 16 images per well were taken. The same procedure is followed with the fourth and fifth plates with the horses 6 and 7 and the antibodies.

The third cell culture plate with the horse 5 is placed in a normal incubator by 38,5°C and 5%CO2. The cells get harvested and counted after 24h and 48h.

The sixth and seventh plates are also placed in the incubator by 38,5°C and 5%CO2. The cells of the sixth plate get harvested and counted after 24h. The cells of the seventh plate get harvested and counted after 48h.

Cell harvesting and counting after 24h and 48h

(Inside the laminar flow)

After 24h the cells out of the first two rows of the third cell culture plate as well as the cells of the whole sixth plate are harvested.

After 48h the cells out of the last two rows of the third cell culture plate as well as the cells of the last three rows of the seventh cell culture plate are harvested.

For doing this, 1,5ml tubes get prepared and labeled equal to each well. The culture plates are taken out of the incubator and stored inside the laminar flow. With a 1000µl pipet the ground of each well is scratched so that the entire cell carpet gets detached. Then the cell suspension gets resuspended up and down carefully and transferred in one of the prepared and labeled 1,5ml tubes. After all cells are harvested and transferred to the prepared and labeled 1,5ml tubes, the cell culture plate gets stored back into the incubator (cell culture plate no. three) or can be thrown away (cell culture plates no. six and seven). Out of each 1,5ml tube 20µl are pipetted into other already prepared and labeled 1,5ml tubes and are used for the cell counting.

The first filled 1,5ml tubes are placed into the centrifuge and get centrifuged for 8min at 2000rpm at 4°C. In the meanwhile, another 2ml cryotubes are prepared and labeled for the supernatant to get collected in. Using a 1000µl pipet, the supernatant from each sample is taken off and stored in the prepared and labeled 2ml cryotubes.

The pellets of the cells harvested from plate no. 3 are provided with 100µl LysesBuffer each and vortexed for 30sec. The pellets of the cells harvested from plate no. 6 and 7 are provided with 50µl LysesBuffer each and vortexed for 30sec.
The supernatant in the cryotubes as well as the pellets with the LysesBuffer are stored at -80°C. The harvested cells are then directly counted with the same procedure already described above.

Antibody staining and counting after 24h via flow cytometer

The cells in the first row of the seventh cell culture plate are harvested after 24h and divided into 2x 500µl. Therefore six 1,5ml tubes are prepared and labeled: two tubes per well are needed.
Then the cells get washed with 1ml of 1% PBS/BSA and centrifuged at RT and 800g for 10min. After the centrifugation of the cells, the supernatant is discarded, and the mixes can be added to the cells.
In the meantime, two antibody-sets with 3 different antibodies in each set get prepared:

- Set 1 = 141µl PBS + 3µl antibodies = 150µl
  - CD4-Fitc = 1µl antibody + 49µl PBS (1:50)
  - CD8-PE = 1µl antibody + 49µl PBS (1:50)
  - CD3-PacBlue = 1µl antibody + 49µl PBS 1:50)
- Set 2 = 105µl PBS + 45µl per antibody = 150µl
  - Monocytes-APC = 15µl antibody + 35µl PBS (1:10)
  - B-cells-PE = 15µl antibody + 35µl PBS (1:10)
  - Interleukin-17-FITC = 15µl antibody + 35µl PBS (1:10)

After the centrifugation of the cells, the supernatant is discarded and 50µl of each set can be given to the cell pellets. After the staining, 1ml of PBS is added to each of the pellets + antibodies to stop the antibody reaction and centrifuged again at RT and 800g for 10min.
After the centrifugation the supernatant with the remaining antibodies is taken off and stored with 400µl PBS in special fax tubes to be measured in the flow cytometer “BD Canto”.

Evaluation of the ”Incucyte S3” live-cell analysis results

After the 24-well-plates with the cells to be evaluated have been removed from the „Incucyte S3“, the stored images must be viewed, and cells are counted by a software. For this purpose, a learning subset of 10 images must be selected and created manually, before automatically all images were analyzed. In this experiment, the following settings were used for the evaluation of the cells:

- Object diameter = 10µm
- Threshold sensitivity = 5
- Texture sensitivity = 5
- Edge sensitivity = 10
- Area = minimal 10µm^2 to maximal 250µm^2

6. Results

The amount of isolated PBMCs differs immensely between the seven different horses. While around 6^7 cells/ml could be extracted from the blood of horse 1, there were significantly fewer cells in horses 3 and 4 with 1,25^6 cells/ml and 7,34^5 cells/ml respectively (figure 2).

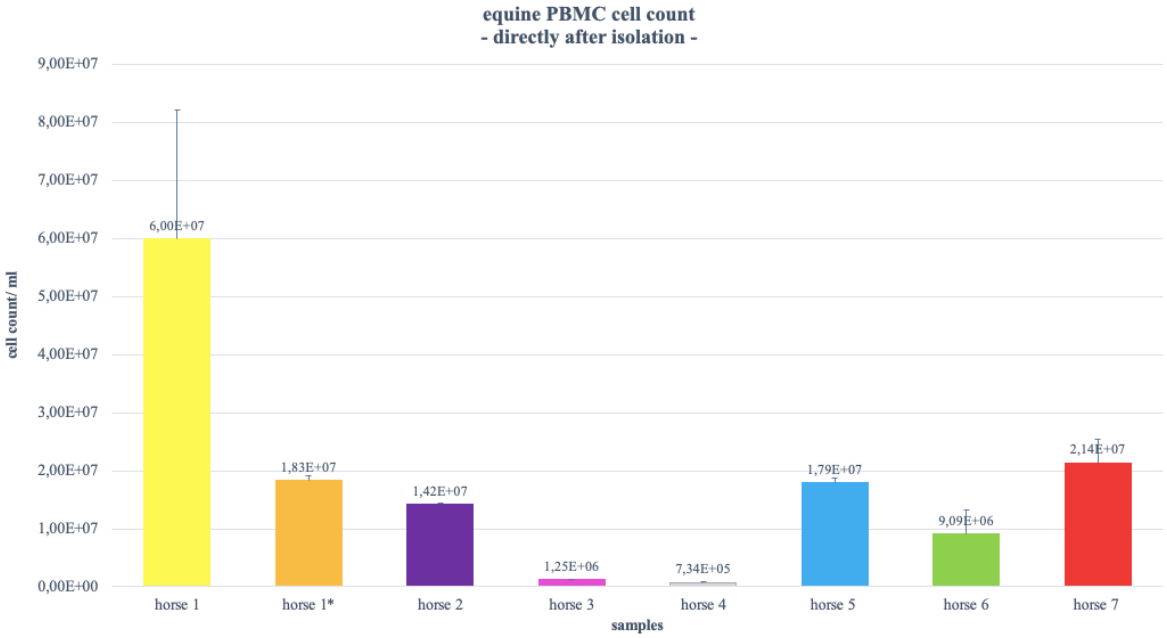

Figure 2: *equine PBMC cell count directly after isolation of all 7 horses*

Figure 3 to 6 represent the course of the cell counts of the horses 1 to 7 measured in the "Incucyte S3". The cell counts of the horses 1 to 2 behaved almost identically (figure 3): in both the negative and positive controls, the cell counts decreased in the FBS and HS serum substitute over time. In comparison, the cell count in the FBS serum substitute with OmniBiotic as treatment remained almost at the same level or decreased only slightly. In contrast, the cell count in the HS serum substitute with OmniBiotic decreased in the same way as with the negative and positive control. The cell counts of the horses 4 and 5 remained at the same level or decreased slightly during the study without an immense difference between the individual treatments of the cells with regard to the serum substitutes used or additives such as ConA or OmniBiotic (figure 4).

Since there were many upward and downward swings on plate 4 (figure 5), the values were averaged again in order to be able to follow a better course (figure 6). In the horses 6 and 7 it can be seen that the cells with OmniBiotic as treatment in all serum substitutes and also all

media showed the greatest tendency to proliferate. The tendency to proliferate is particularly noticeable with the medium RPMI and FC1, closely followed by RPMI and FBS. The medium DMEM with the individual HS serum substitute on the other hand tended to decrease the cell numbers (figure 6).

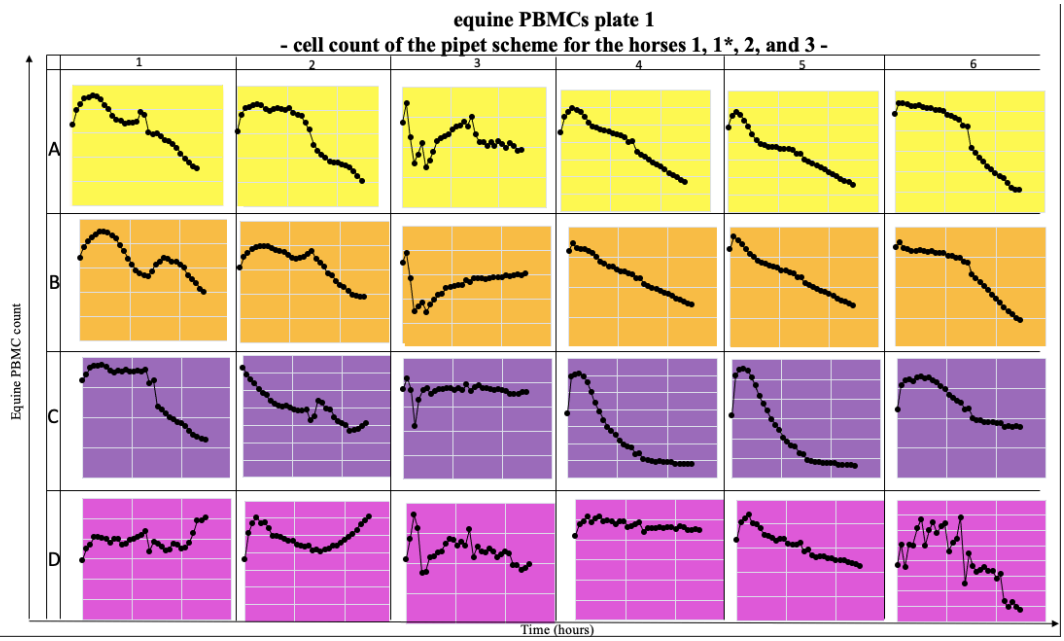

Figure 3: *plate 1 - equine PBMC cell count of the pipet scheme for the horses 1, 1*, 2, 3*

Row A shows the course of the cells counts of horse 1 over a period of 124h with the different serum substitutes and treatments. Row B shows the cell count from horse 1, row C from horse 2 and row D from horse 3.*
Column 1 represents the negative control with FBS for each horse, column 2 FBS + ConA, column 3 FBS + OB, column 4 the negative control with HS, column 5 HS + ConA and column 6 HS + OB.

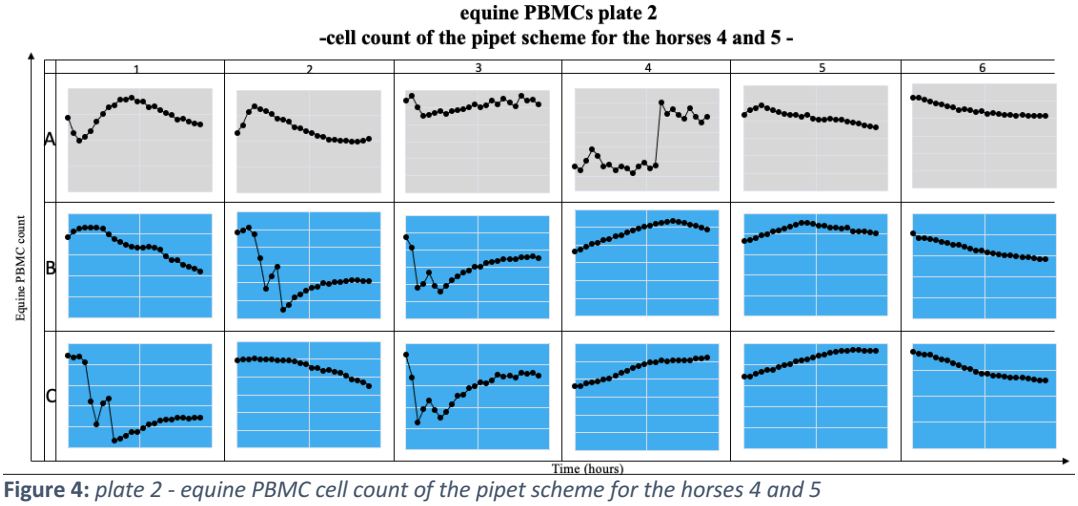

Figure 4: *plate 2 - equine PBMC cell count of the pipet scheme for the horses 4 and 5*

Row A shows the course of the cell counts of horse 4 over a period of 92h. Row B and C show the cell counts from horse 5.
Column 1 represents the negative control with FBS for each horse, column 2 FBS + ConA, column 3 FBS + OB, column 4 the negative control with HS, column 5 HS + ConA and column 6 HS + OB.

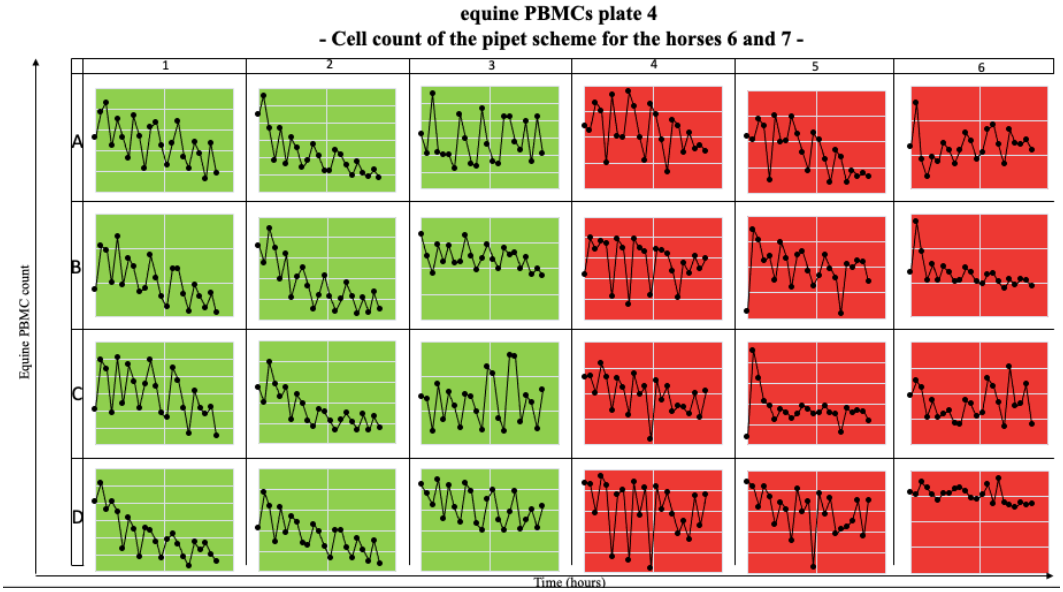

Figure 5: *plate 4 - equine PBMC cell count of the pipet scheme for the horses 6 and 7*

The green fields represent the cell counts of horse 6, the red fields those of horse 7 over a period of 88h.
Row A contains RPMI as medium and FBS as serum substitute, row B RPMI + HS, row C RPMI + FC1 and row D DMEM + HS for each of the two horses.
The columns 1 and 4 show the negative controls for each horse without any treatment, columns 2 and 5 contain ConA as a positive control and the columns 3 and 6 contain OB.

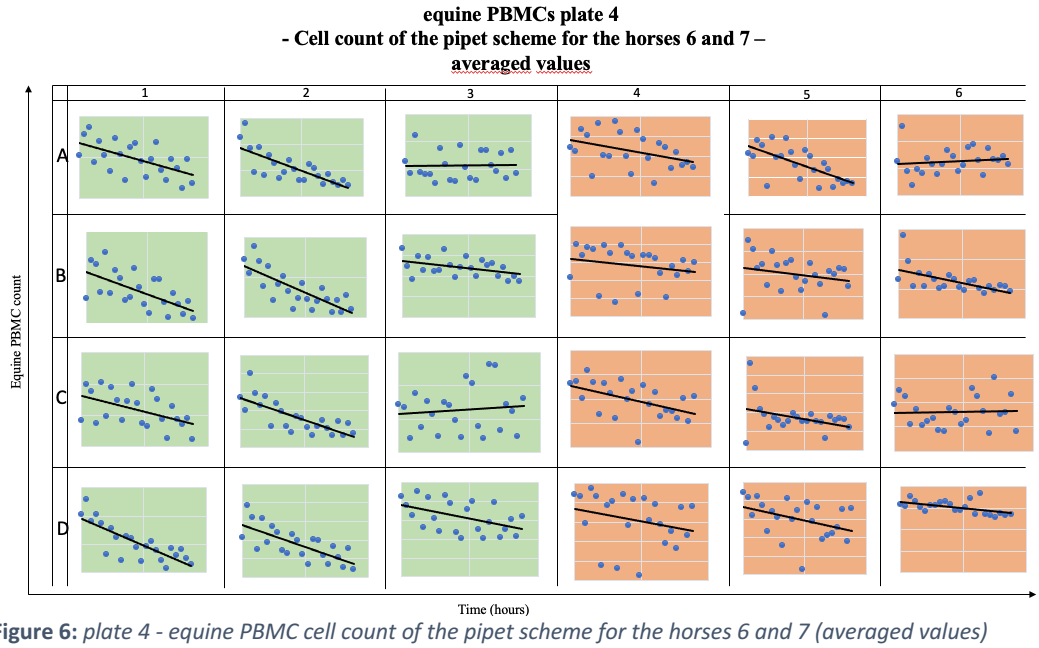

Figure 6: *plate 4 - equine PBMC cell count of the pipet scheme for the horses 6 and 7 (averaged values)*

The green fields represent the cell counts of horse 6, the red fields those of horse 7 over a period of 88h.
Row A contains RPMI as medium and FBS as serum substitute, row B RPMI + HS, row C RPMI + FC1 and row D DMEM + HS for each of the two horses.
The columns 1 and 4 show the negative controls for each horse without any treatment, columns 2 and 5 contain ConA as a positive control and the columns 3 and 6 contain OB.
The values correspond to those in figure 5 but have been averaged to show a better progression.

The quantity of living PBMCs from horse 5 decreased after 48h in the incubator compared to 24h, regardless of the serum the cells were treated with or the positive/ negative control and the OmniBiotic as additives were used (figure 7).

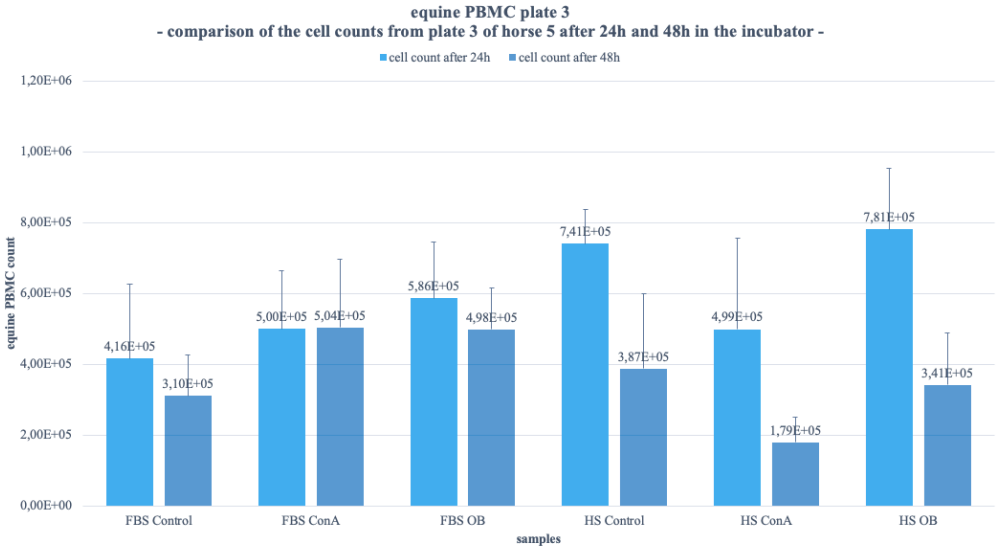

Figure 7: *plate 3 - comparison of the cell counts of horse 5 after 24h and 48h in the incubator*
The light blue bars represent the amount of living PBMCs after 24h in the incubator with the different serum substitutes and treatments. The dark blue bars also represent the amount of living PBMCs with the same different additives, but after 48h in the incubator.

The quantity of living PBMCs from horse 7 increased after 48h in the incubator compared to 24h, regardless of the medium or serum the cells were treated with or which additive as a positive/ negative control and the OmniBiotic was used (figure 8).

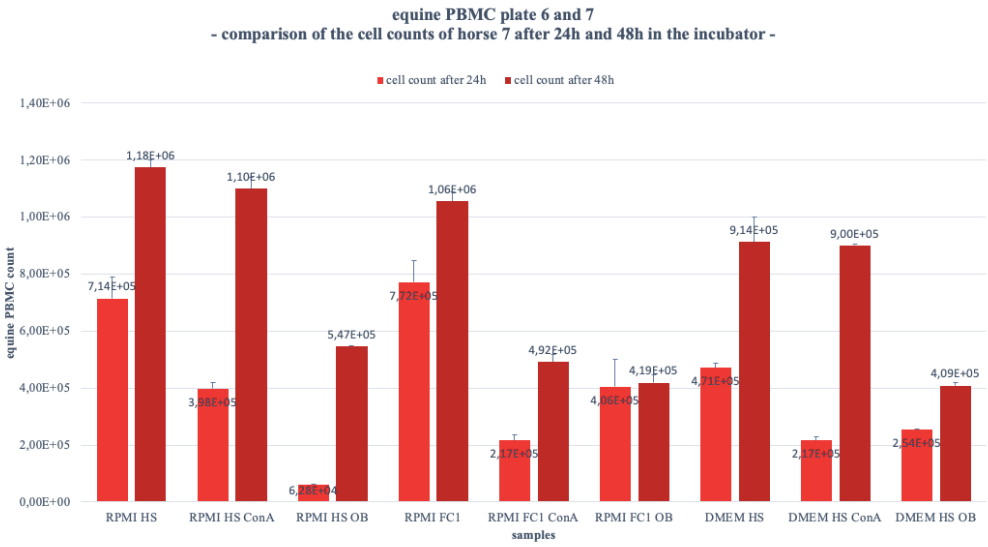

Figure 8: *plate 6 and 7 - comparison of the cell counts of horse 7 after 24h and 48h in the incubator*
The light red bars represent the amount of living PBMCs after 24h in the incubator with the different mediums, serum substitutes and treatments. The dark red bars also represent the amount of living PBMCs with the same different additives, but after 48h in the incubator.

Diagrams 9 to 14 show the course of the cell count in both different serum substitutes FBS and HS coupled with the treatments of the negative and positive control as well as with OmniBiotic. Apart from horse 5, the cell counts dropped consistently over time, except for the treatment with OmniBiotic in the FBS serum substitute (figure 13): here, the cell counts remained at least stable or even increased in some cases, even if only by a few cells.

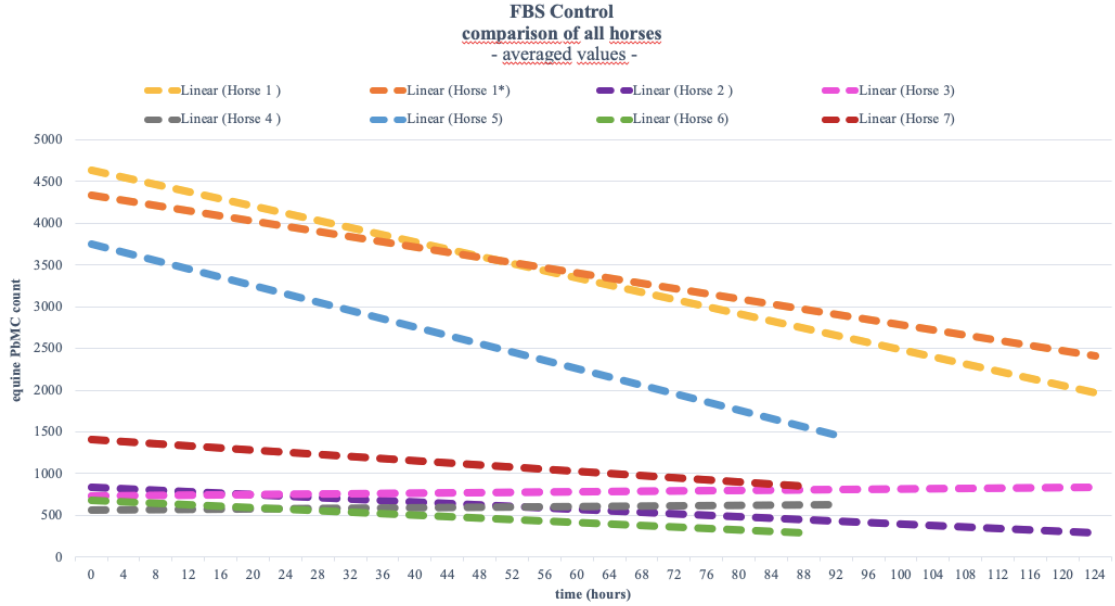

Figure 9: *comparison of the cell counts of all horses in FBS alone (=negative control)*
The lines of the individual horses show the course of the cell count over 124h. The cells were only in FBS medium which served as a negative control.

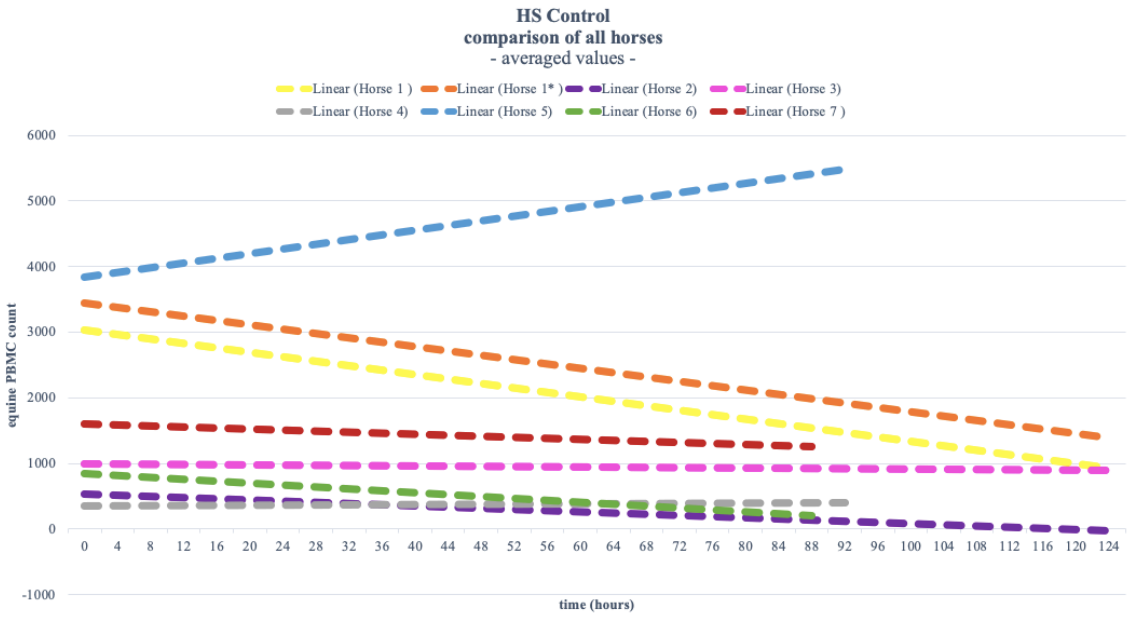

Figure 10: *comparison of the cell counts of all horses in HS alone (=negative control)*
The lines of the individual horses show the course of the cell count over 124h. The cells were only in HS which served as a negative control.

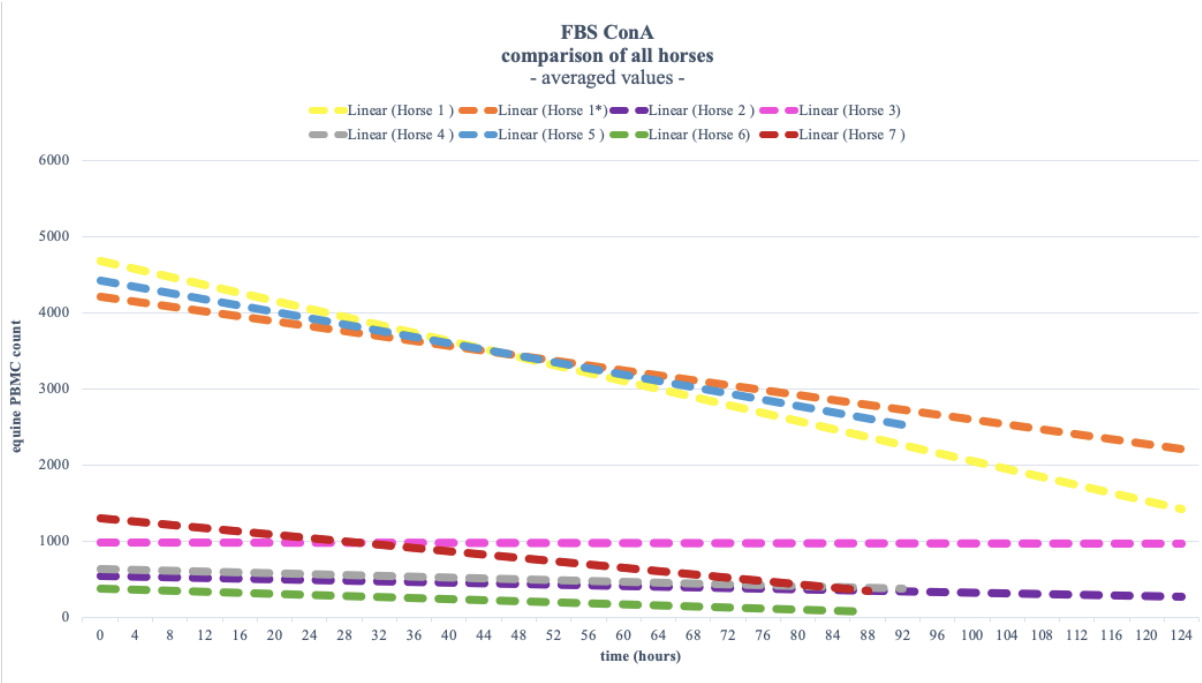

Figure 11: *comparison of the cell counts of all horses in FBS and ConA (=positive control)*
The lines of the individual horses show the course of the cell count over 124h. The cells were in FBS and ConA which served as a positive control.

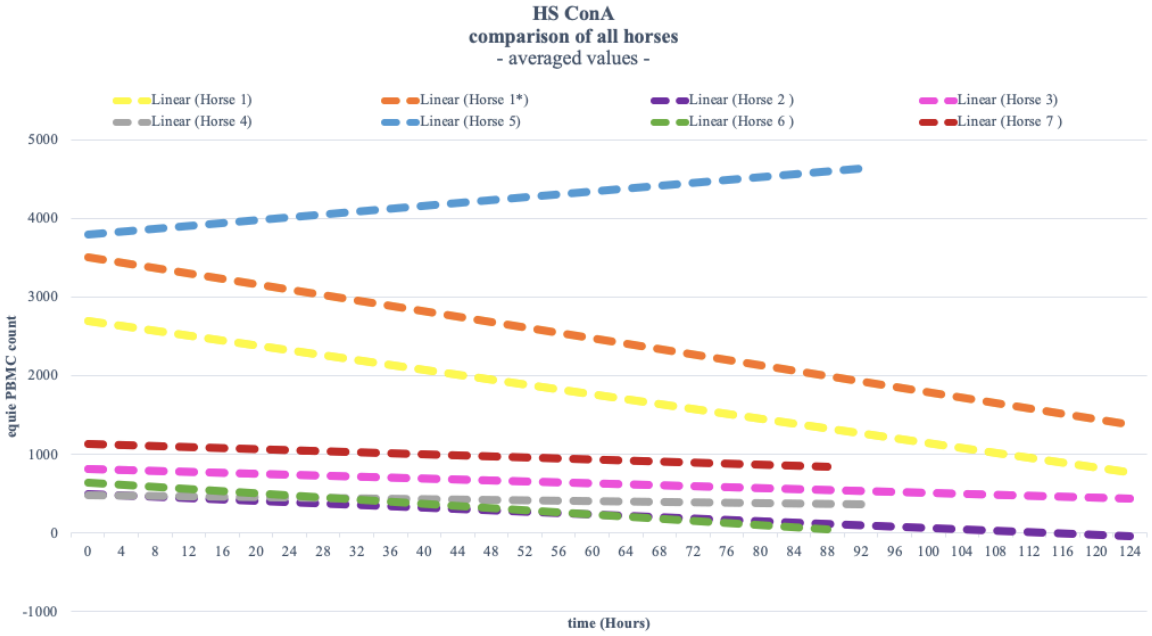

Figure 12: *comparison of the cell counts of all horses in HS and ConA (=positive control)*
The lines of the individual horses show the course of the cell count over 124h. The cells were in HS and ConA which served as a positive control.

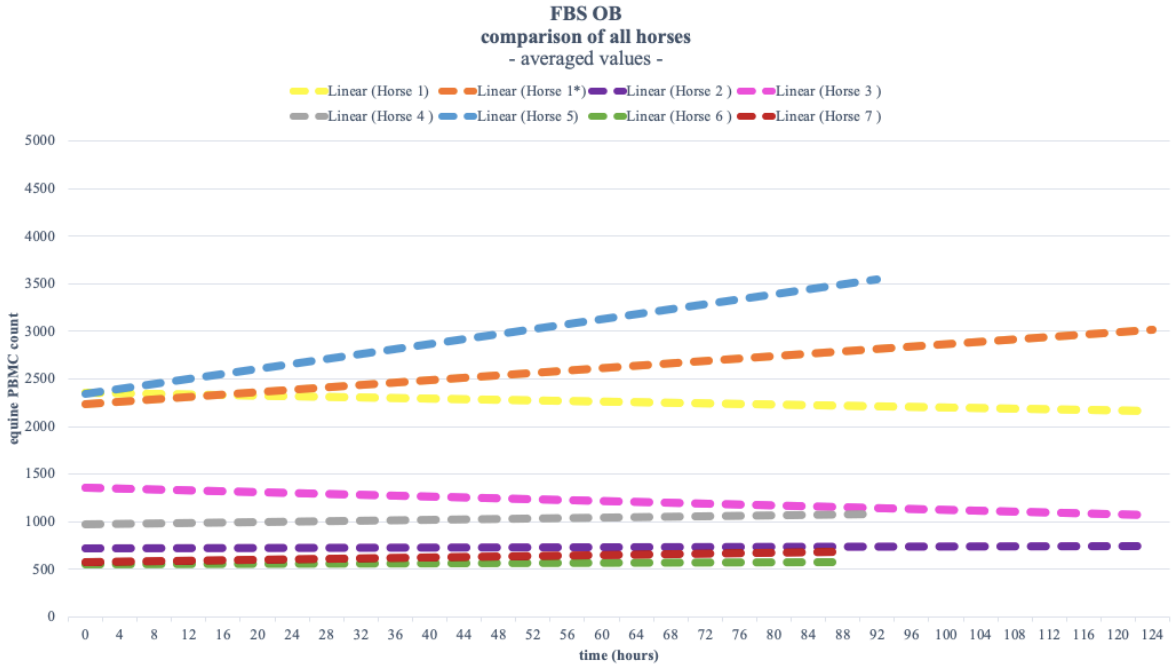

Figure 13: *comparison of the cell counts of all horses in FBS and OB*
The lines of the individual horses show the course of the cell count over 124h. The cells were in FBS and OB.

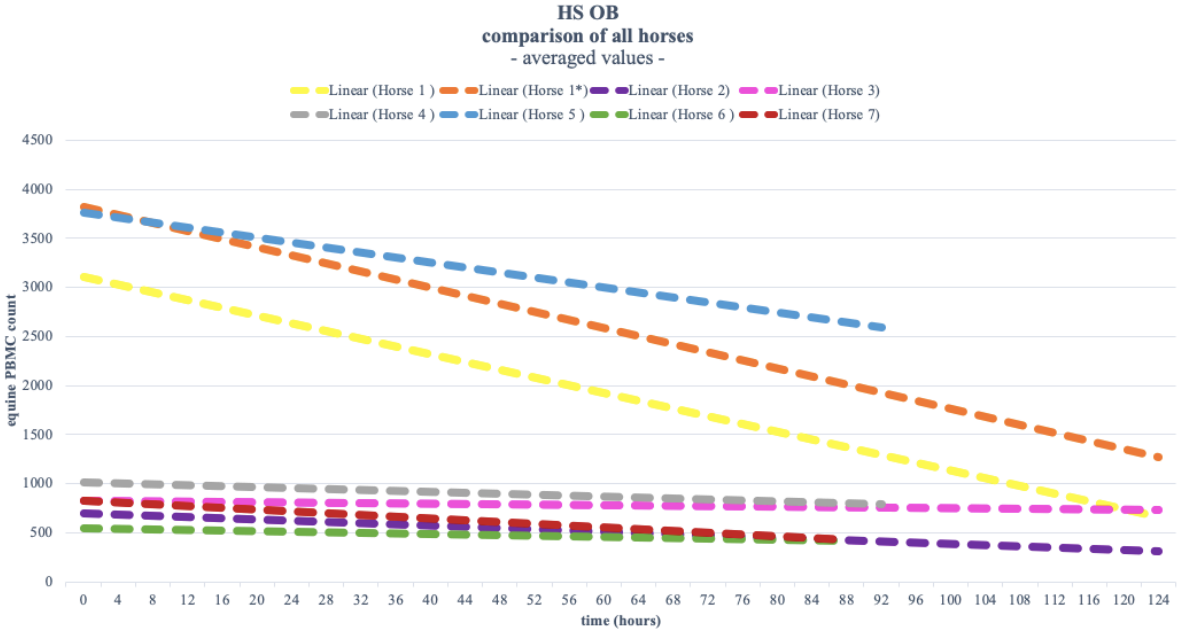

Figure 14: *comparison of the cell counts of all horses in HS and OB*
The lines of the individual horses show the course of the cell count over 124h. The cells were in HS and OB.

Following the comparisons from diagram 9 to 14, the boxplots in diagrams 15 to 17 also show the cell counts in the two different serum substitutes with the three different treatments (negative without any additive, positive control with ConA as an additive and OmniBiotic) for horse 1 to 5.

Without any treatment, except for horse 3 and 5, the cell count of the PBMC's was always higher with the FBS than with the horse serum and the range of the cell count was similar in both sera (figure 15). The same statement can be made about the treatment with ConA as a positive control (figure 16). On the other hand, the graph in figure 17 shows that the cell counts with OmniBiotic as treatment in the horse serum tended to be higher than those with FBS as serum substitute, especially when looking at horses 1 and 1* in comparison to figure 15 and 16. However, the values of horse 3 to 5 contradict this result again, as there is hardly any difference to the treatment from figure 15 and 16 noticeable.

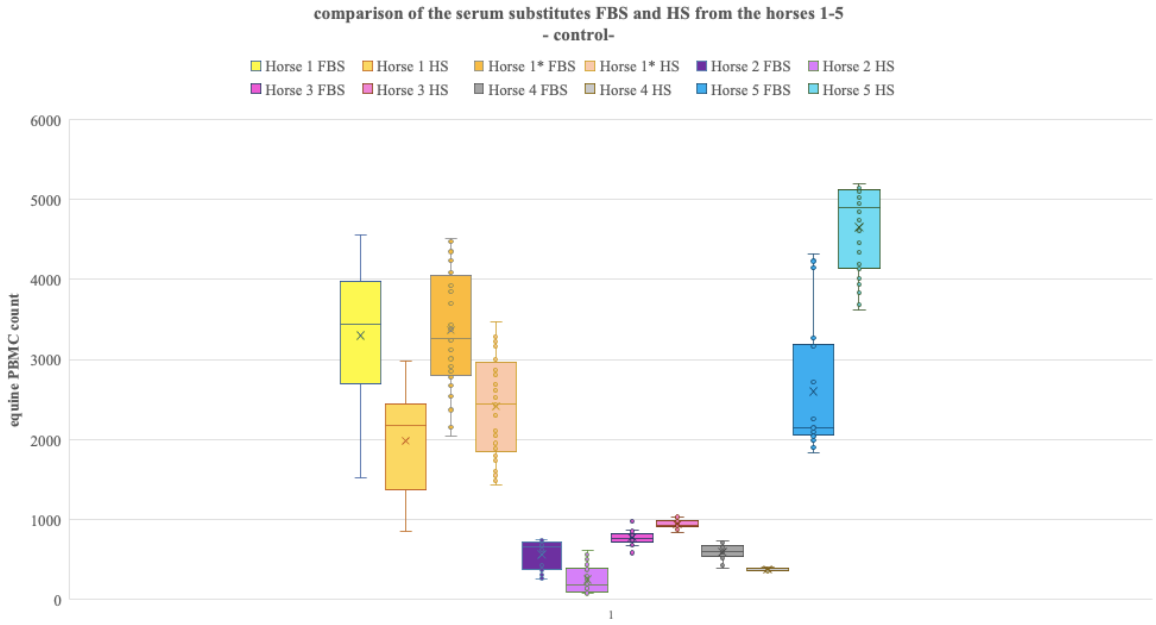

Figure 15: *comparison of the cell counts from the horses 1-5 in FBS and HS alone (=negative control)*
The colored box plots assigned to the horses 1-5 show the distribution of the cell count in the comparison of the two serum substitutes FBS and HS.

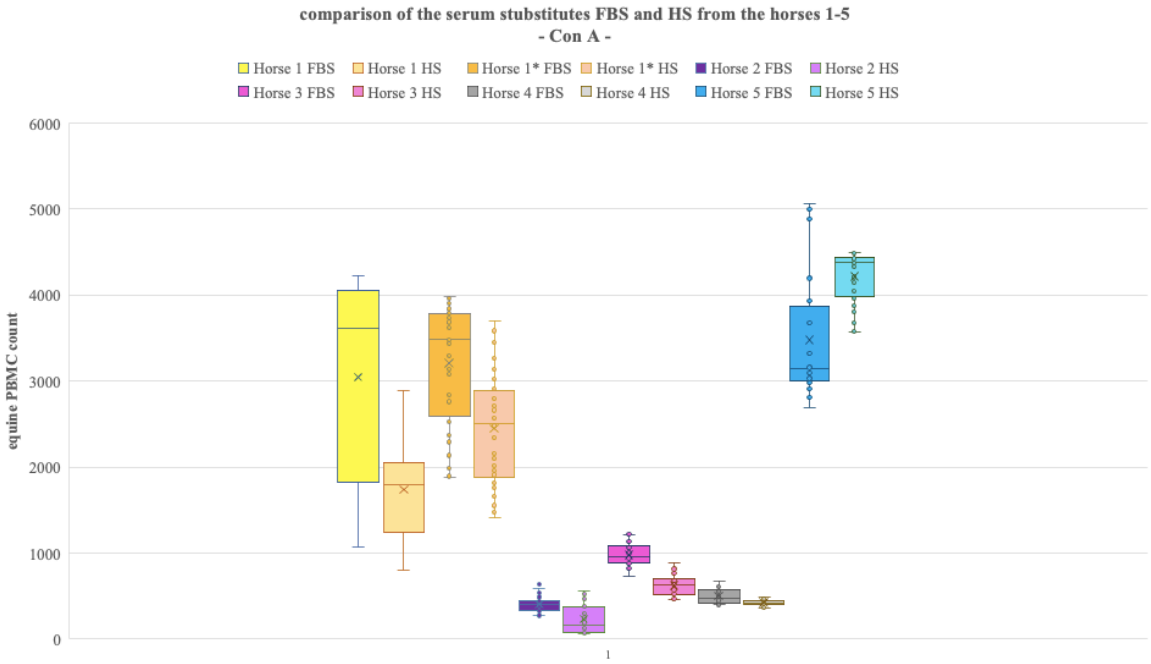

Figure 16: *comparison of the cell counts from the horses 1-5 in the serum substitutes FBS and HS and ConA (=positive control)*
The colored box plots assigned to the horses 1-5 show the distribution of the cell count in the comparison of the two serum substitutes FBS and HS and ConA as an additive.

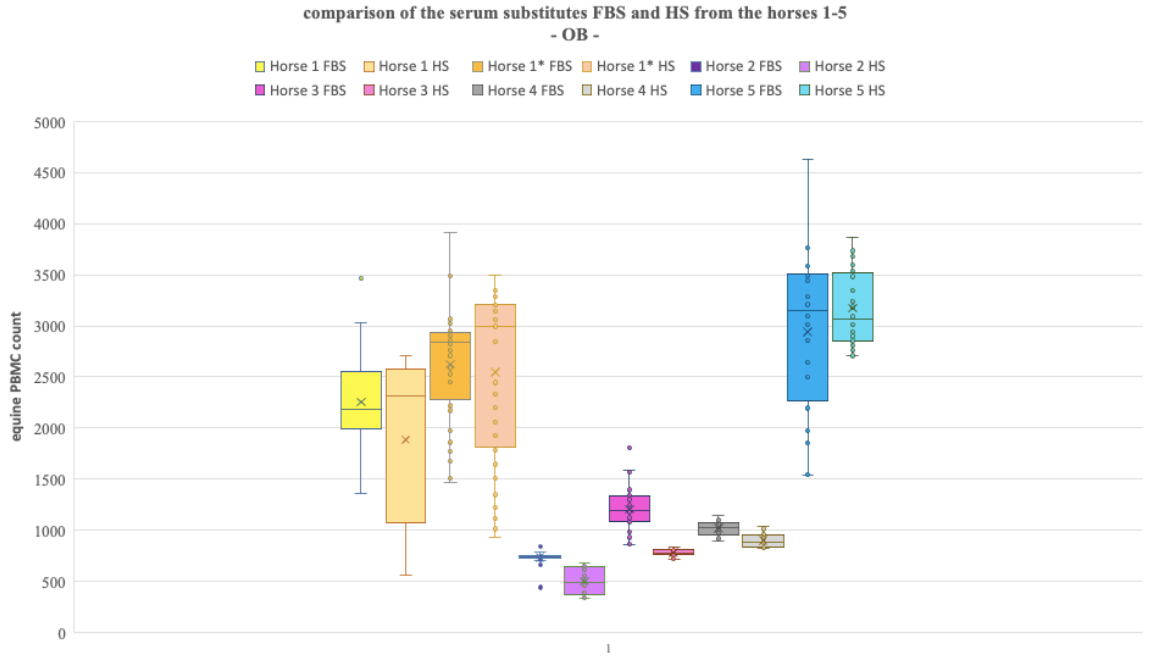

Figure 17: *comparison of the cell counts from the horses 1-5 in the serum substitutes FBS and HS and OB*
The colored box plots assigned to the horses 1-5 show the distribution of the cell count in the comparison of the two serum substitutes FBS and HS and OB as an additive.

Figures 18, 19 and 20 show boxplots of the equine PBMC cell counts of horse 6 and 7 in all three different serum substitutes FBS vs. HS vs FC1 and the three different treatments with negative control vs. positive control ConA vs OmniBiotic. Comparing the cell counts of the three different serum substitutes, it can be seen that within the negative control, the cell counts within a horse are in the same range, regardless which serum substitute was used (figure 18). The PBMCs from horse 6 were more comfortable in the FC1 serum substitute whereas the cells from horse 7 preferred the horse serum. However, the cell numbers in comparison with ConA as a treatment are already further apart and it can be seen that the PBMCs of horse 6 and 7 felt most comfortable in the combination with HS and ConA, the cells of horse 6 in the combination with FBS and ConA and the PBMCs of horse 7 with FC1 and ConA felt most uncomfortable (figure 19). Figure 20 shows, that with OmniBiotic as a treatment, the cell count was highest in the combination with FC1 as the serum substitute in both horses.

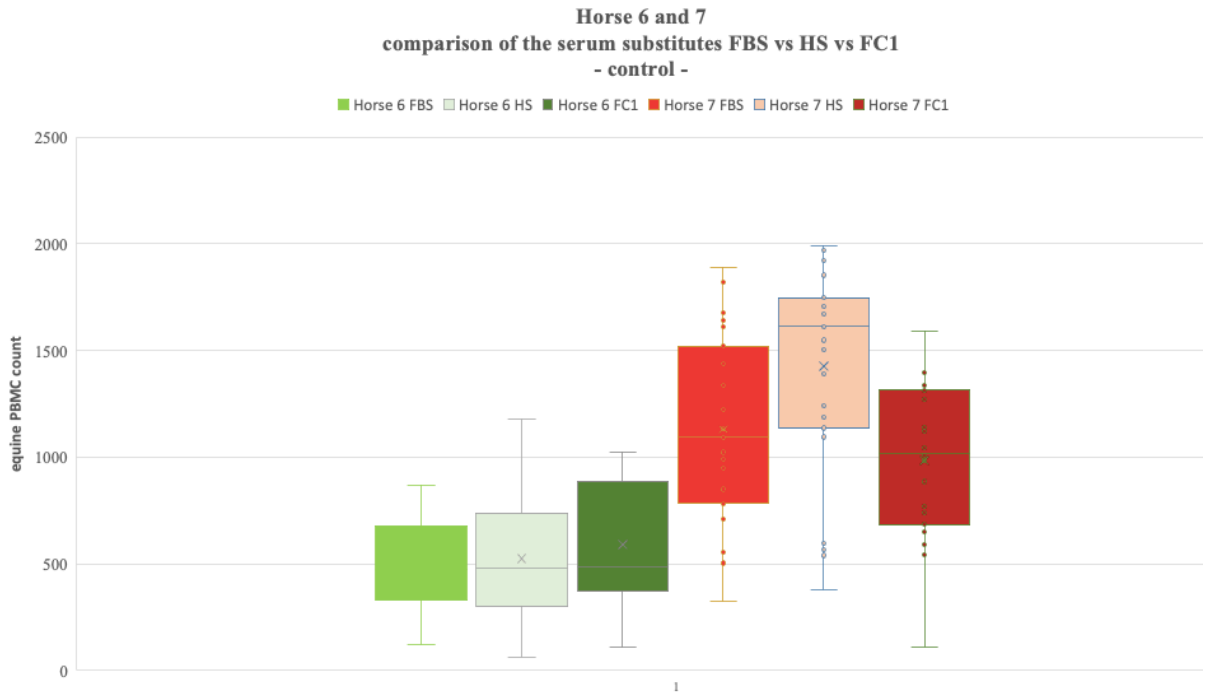

Figure 18: *comparison of the cell counts in the three different serum substitutes FBS vs. HS vs FC1 alone (=negative control) The colored box blots assigned to the horses 6 and 7 show the distribution of the cell counts in comparison of the three serum substitutes FBS and HS and FC1.*

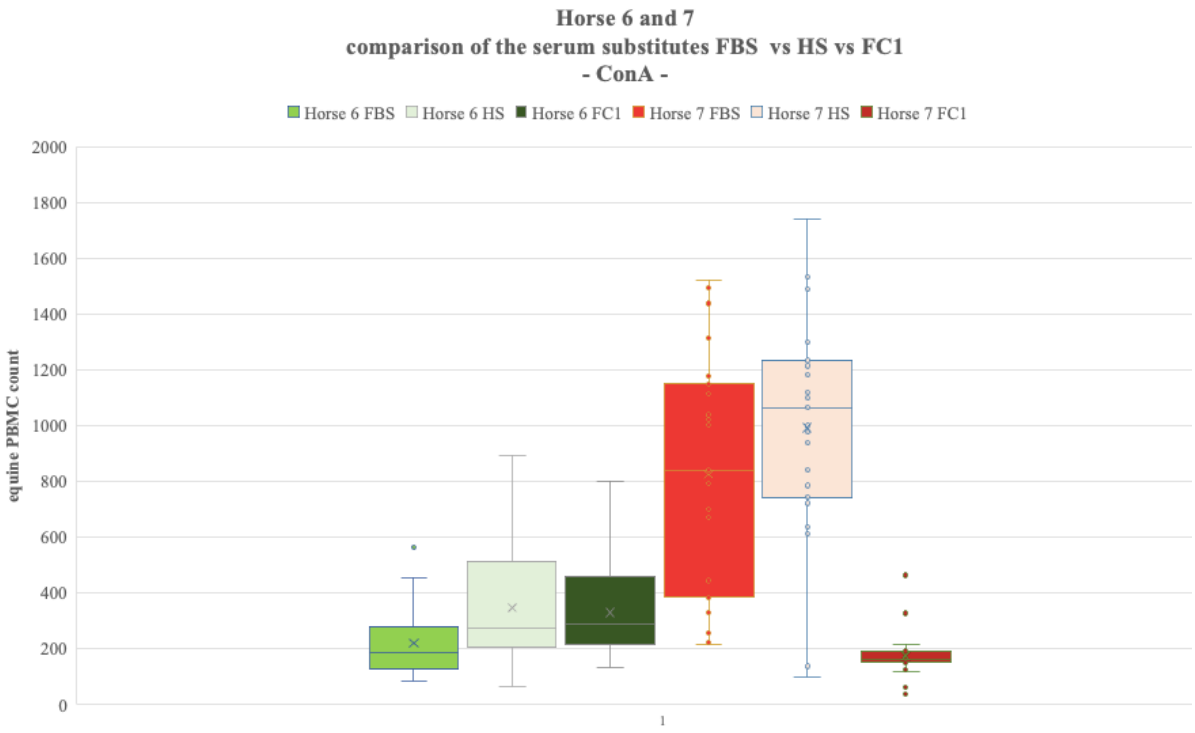

Figure 19: *comparison of the cell counts in three different serum substitutes FBS vs HS vs FC1 and ConA (=positive control)*
The colored box blots assigned to the horses 6 and 7 show the distribution of the cell counts in comparison of the three serum substitutes FBS and HS and FC1 as well as with ConA as an additive.

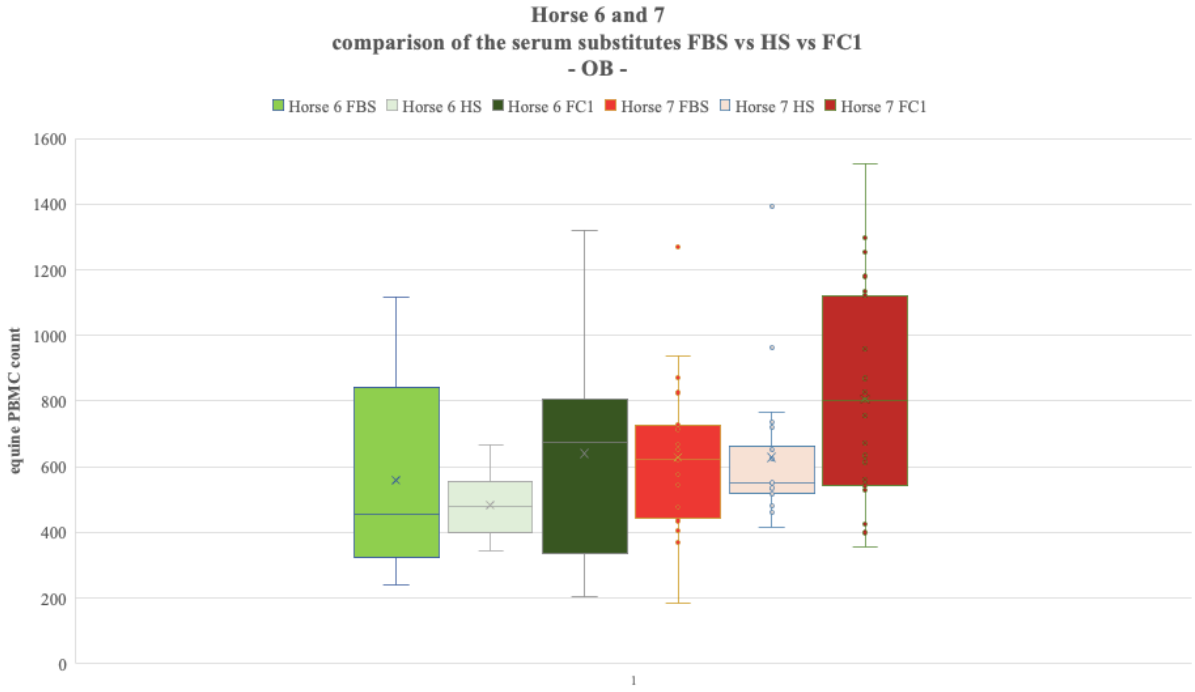

Figure 20: *comparison of the cell counts in the three different serum substitutes FBS vs. HS .vs FC1 with OB*
The colored box blots assigned to the horses 6 and 7 show the distribution of the cell counts in comparison of the three serum substitutes FBS and HS and FC1 as well as with OB as an additive.

Furthermore, figures 21, 22 and 23 compare the cell count of PBMCs in RPMI and DMEM medium in combination with HS as serum substitute and the three different mediums. In all three figures it is clear, that the cell count in the RPMI medium was significantly higher than with the DMEM medium, regardless of which horses' cells or which treatment the cells were subjected to.

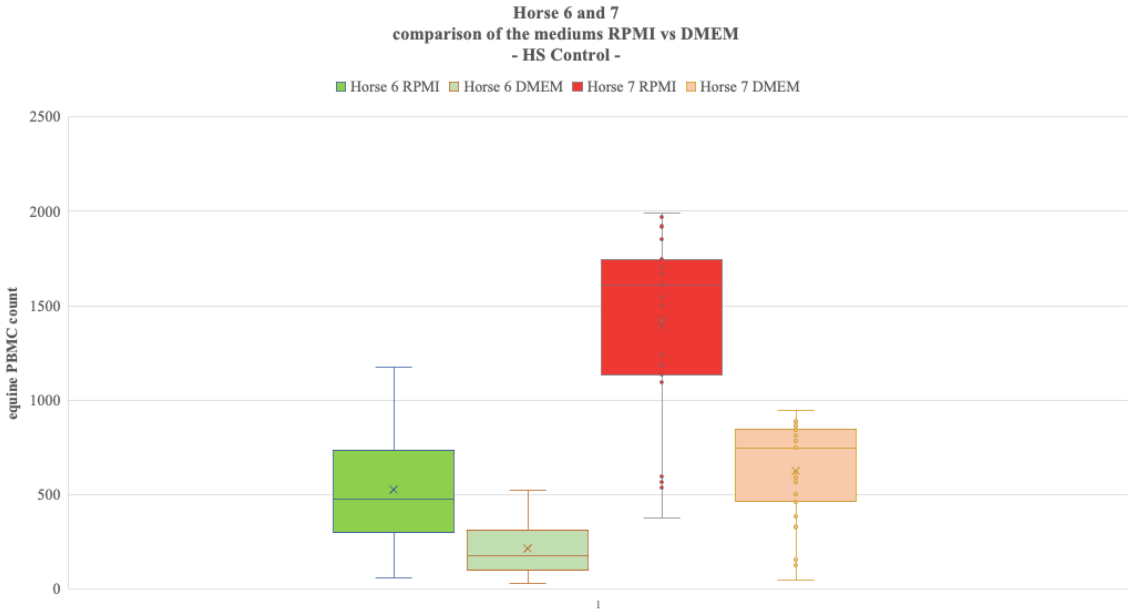

Figure 21: *comparison of the cell counts in two different mediums RPMI vs DMEM alone (=negative control)*
The colored box blots assigned to the horses 6 and 7 show the distribution of the cell count in comparison with the two different media RPMI and DMEM as well as the serum substitute HS.

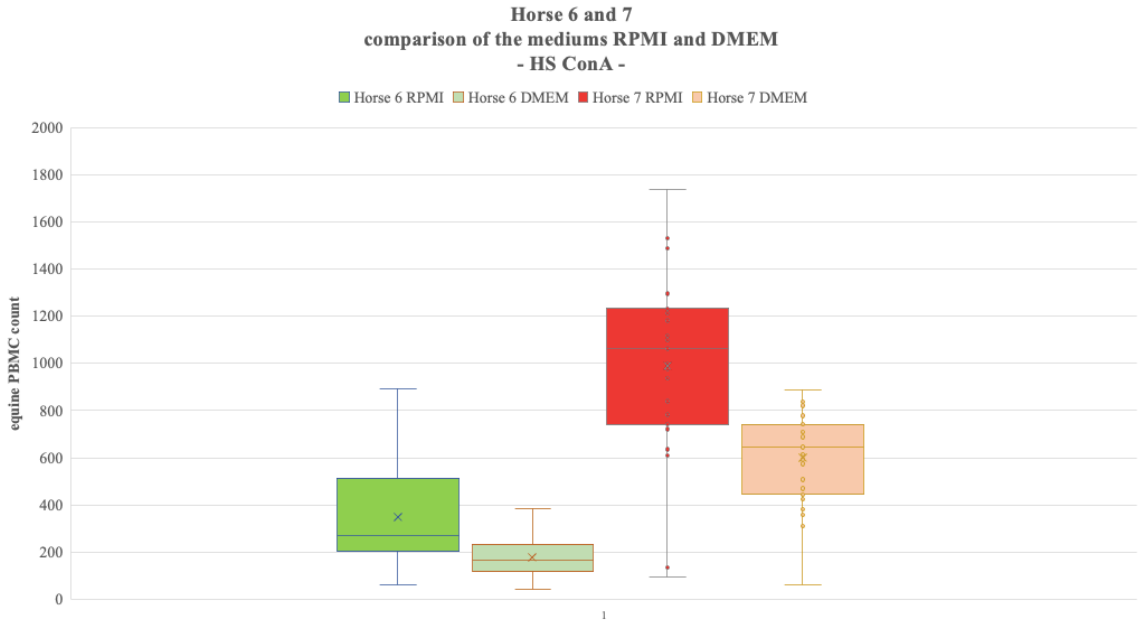

Figure 22: *comparison of the cell counts in different mediums RPMI vs DMEM and ConA (=positive control)*
The colored box blots assigned to the horses 6 and 7 show the distribution of the cell count in comparison with the two different media RPMI and DMEM as well as the serum substitute HS and ConA as an additive.

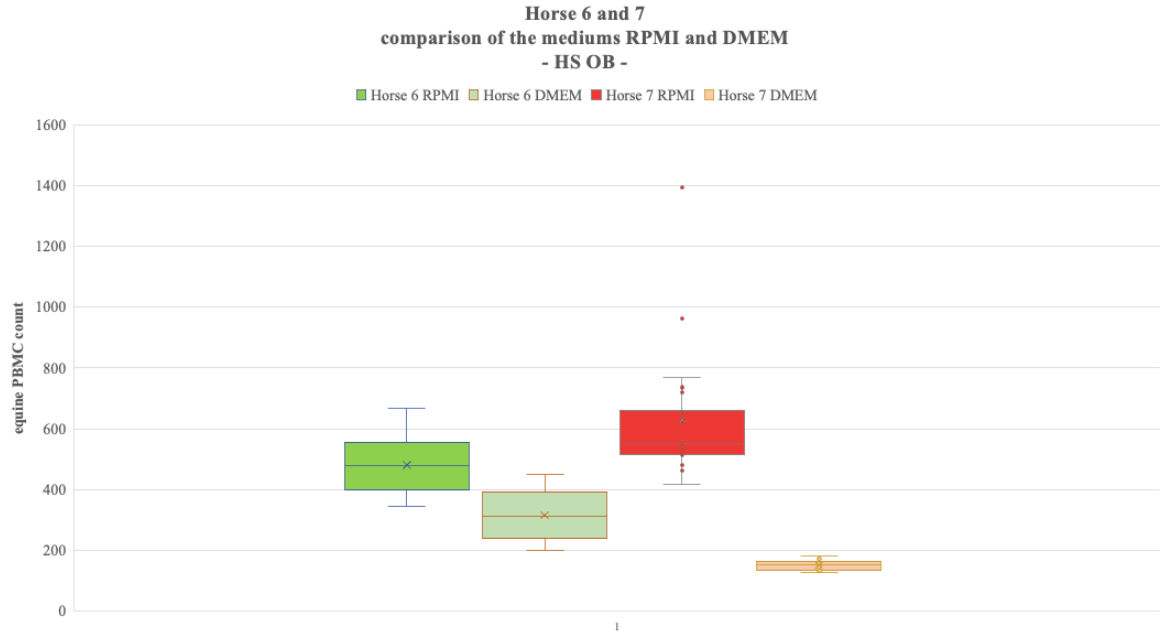

Figure 23: *comparison of the cell counts in different mediums RPMI vs DMEM and OB*
The colored box blots assigned to the horses 6 and 7 show the distribution of the cell count in comparison with the two different media RPMI and DMEM as well as the serum substitute HS and OB as an additive.

Figures 24 to 30 show the cell counts of PBMCs in each horse with the comparison between the two different serum substitutes FBS and HS used as well as with the different treatments. The PBMCs of horse 1 did better in the FBS serum substitute than in the HS serum substitute and the most cells could be counted in the negative control and the positive control with ConA (figure 24). The difference between the two serum substitutes could also be detected with horse 2, but the highest cell counts were obtained with OB in FBS (figure 25). The same can be said about the results the horse 3 and 4, where the highest cell counts of horse 4, similar to horse 1, were counted as negative control in FBS (figure 26 and 27). The presentation of horse 5 to 7 in figures 28 to 30 represents a deviation from the preferences of the cells in the different serum substitutes and treatment combinations described above. The PBMCs of horse 5 and 7 were more comfortable in HS as serum substitutes than in FBS.

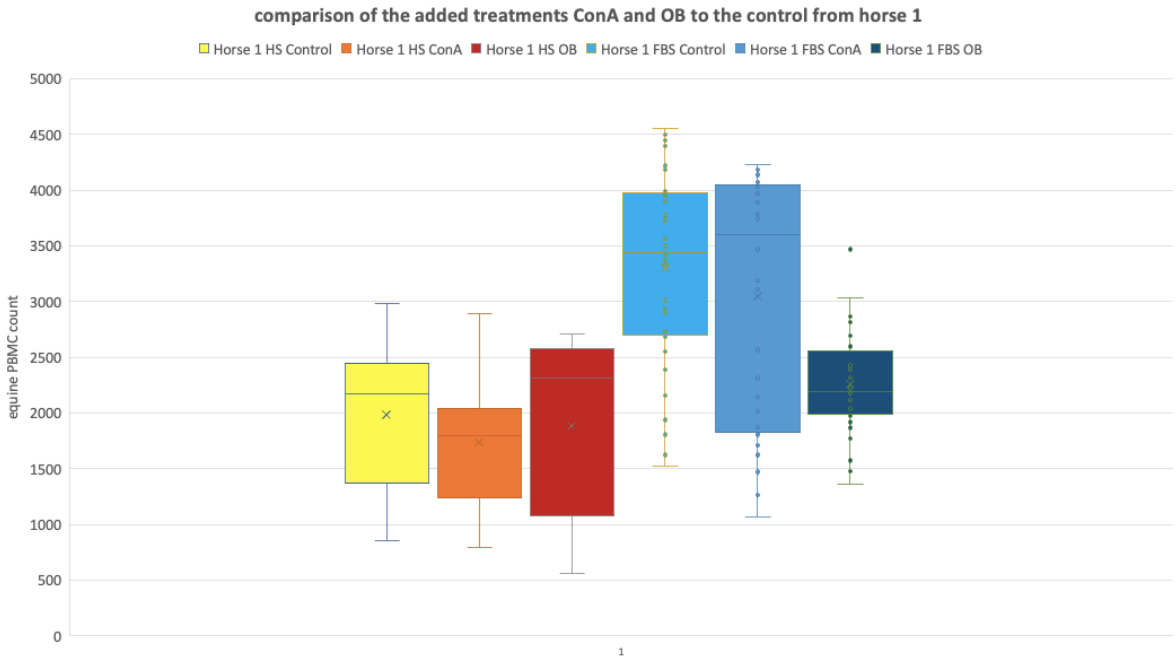

Figure 24: *comparison from horse 1 with the two different serum substitutes FBS and HS and different treatments with ConA and OB and the negative control*

The colored box blots assigned to the serum substitutes and additives used show the distribution of the cell count in comparison with the two different serum substitutes HS and FBS used as well as the different additives ConA and OB.

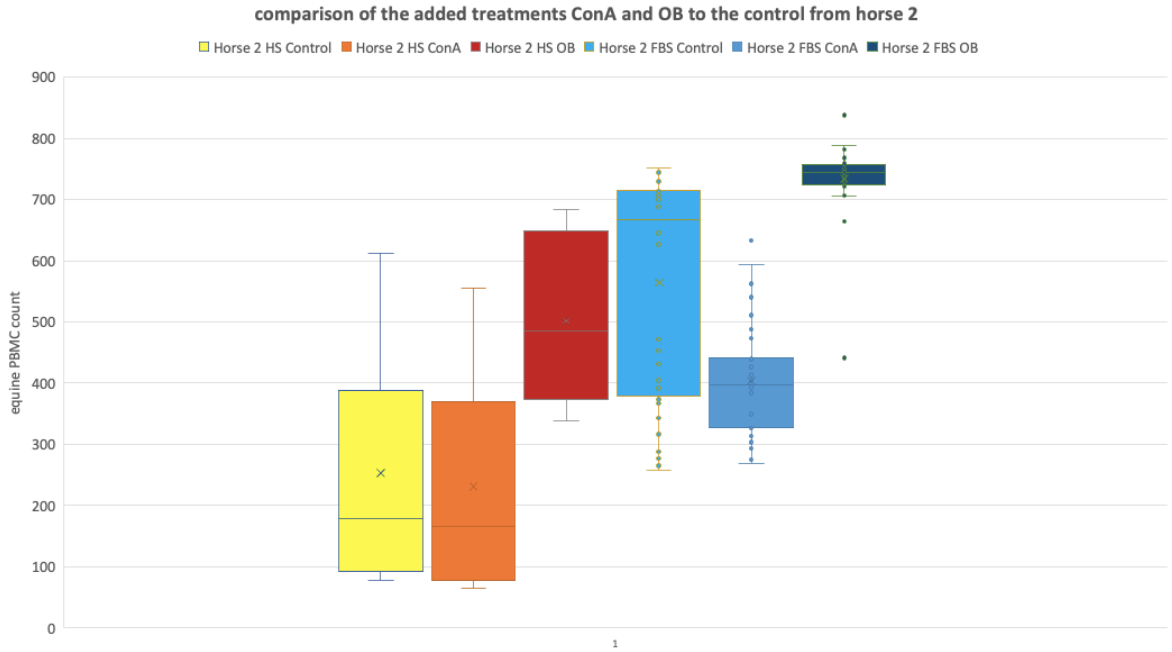

Figure 25: *comparison from horse 2 with the two different serum substitutes FBS and HS and different treatments with ConA and OB and the negative control*

The colored box blots assigned to the serum substitutes and additives used show the distribution of the cell count in comparison with the two different serum substitutes HS and FBS used as well as the different additives ConA and OB.

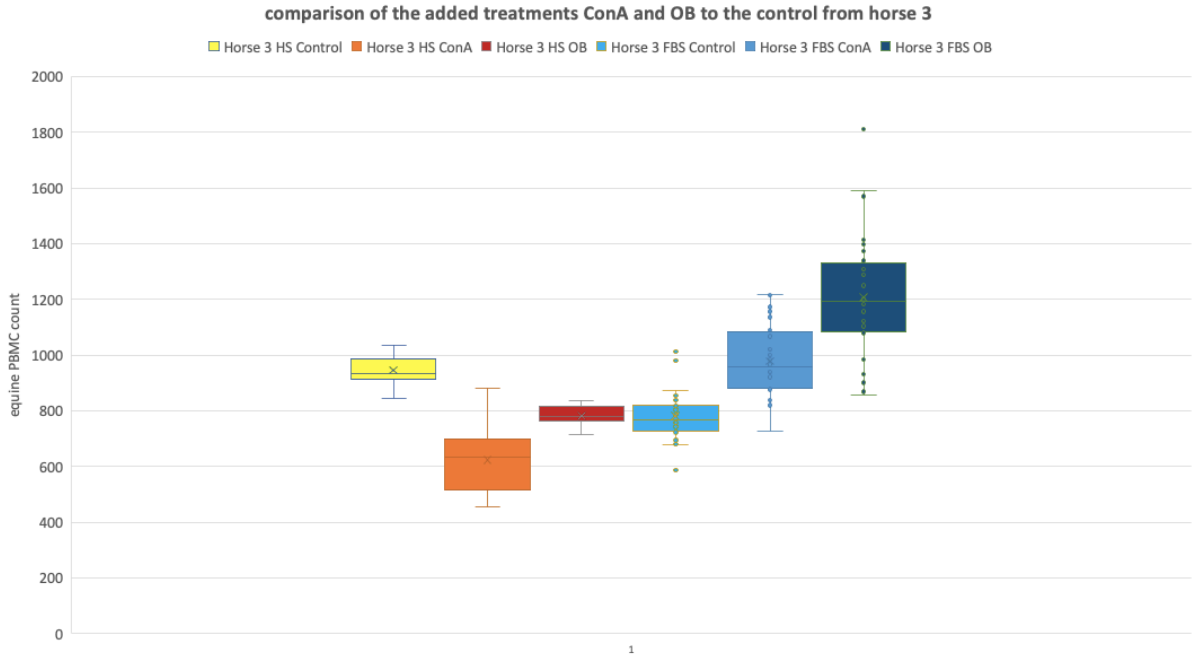

Figure 26: *comparison from horse 3 with the two different serum substitutes FBS and HS and different treatments with ConA and OB and the negative control*

The colored box blots assigned to the serum substitutes and additives used show the distribution of the cell count in comparison with the two different serum substitutes HS and FBS used as well as the different additives ConA and OB.

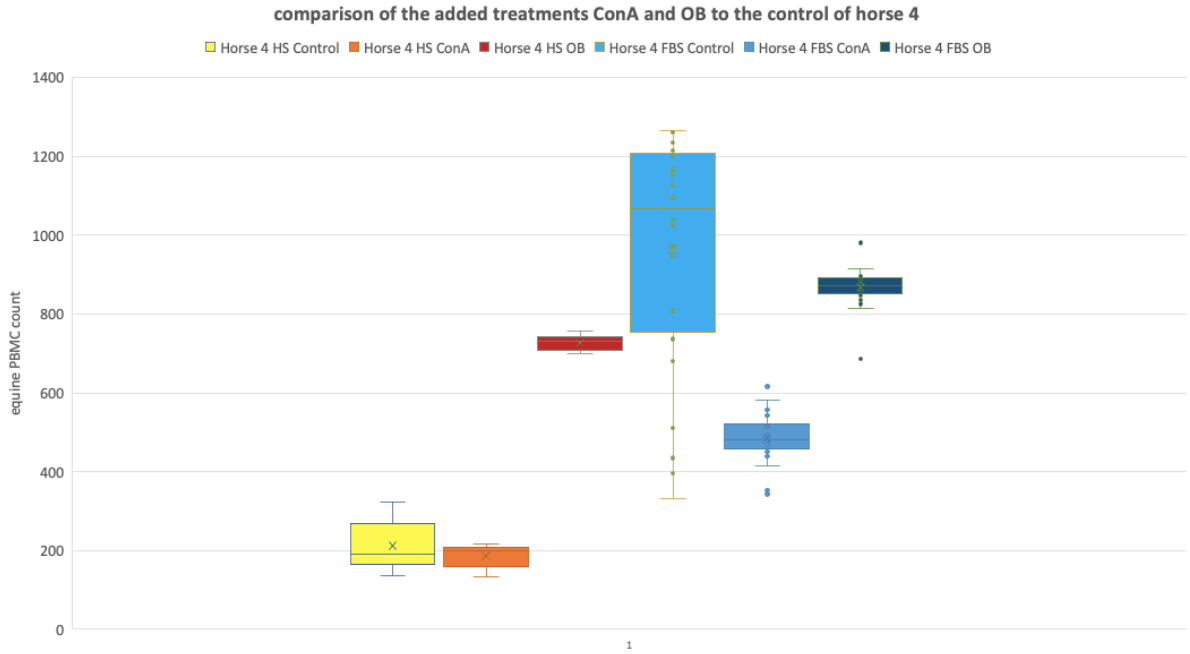

Figure 27: *comparison from horse 4 with the two different serum substitutes FBS and HS and different treatments with ConA and OB and the negative control*

The colored box blots assigned to the serum substitutes and additives used show the distribution of the cell count in comparison with the two different serum substitutes HS and FBS used as well as the different additives ConA and OB.

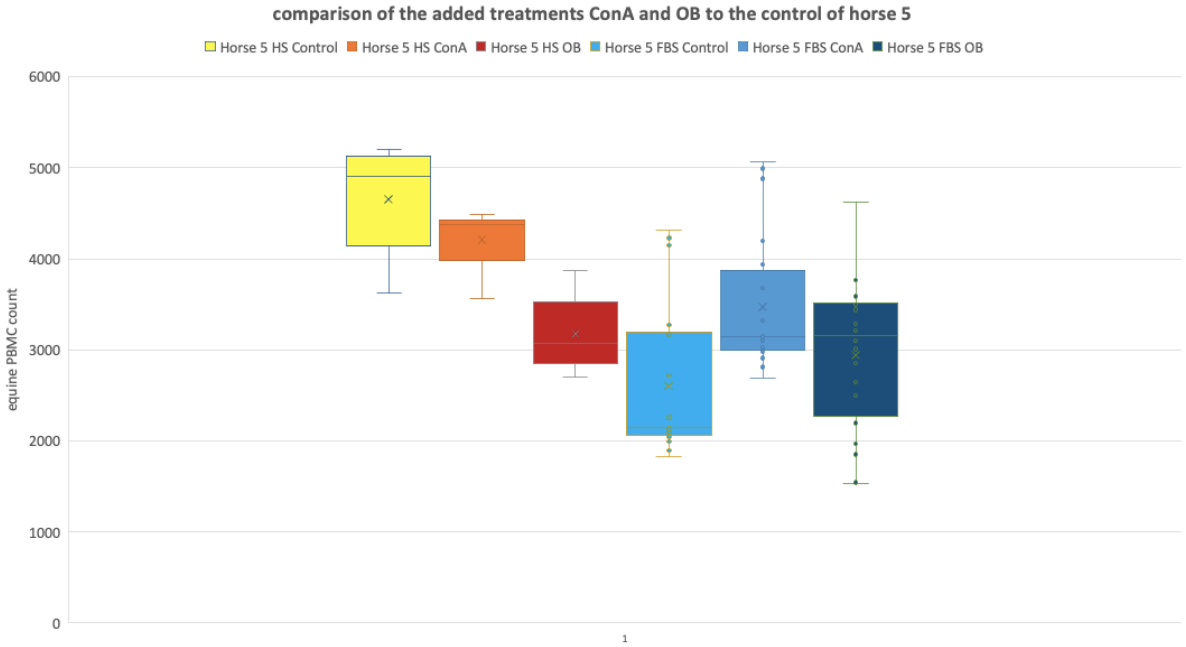

Figure 28: *comparison from horse 5 with the two different serum substitutes FBS and HS and different treatments with ConA and OB and the negative control*

The colored box blots assigned to the serum substitutes and additives used show the distribution of the cell count in comparison with the two different serum substitutes HS and FBS used as well as the different additives ConA and OB.

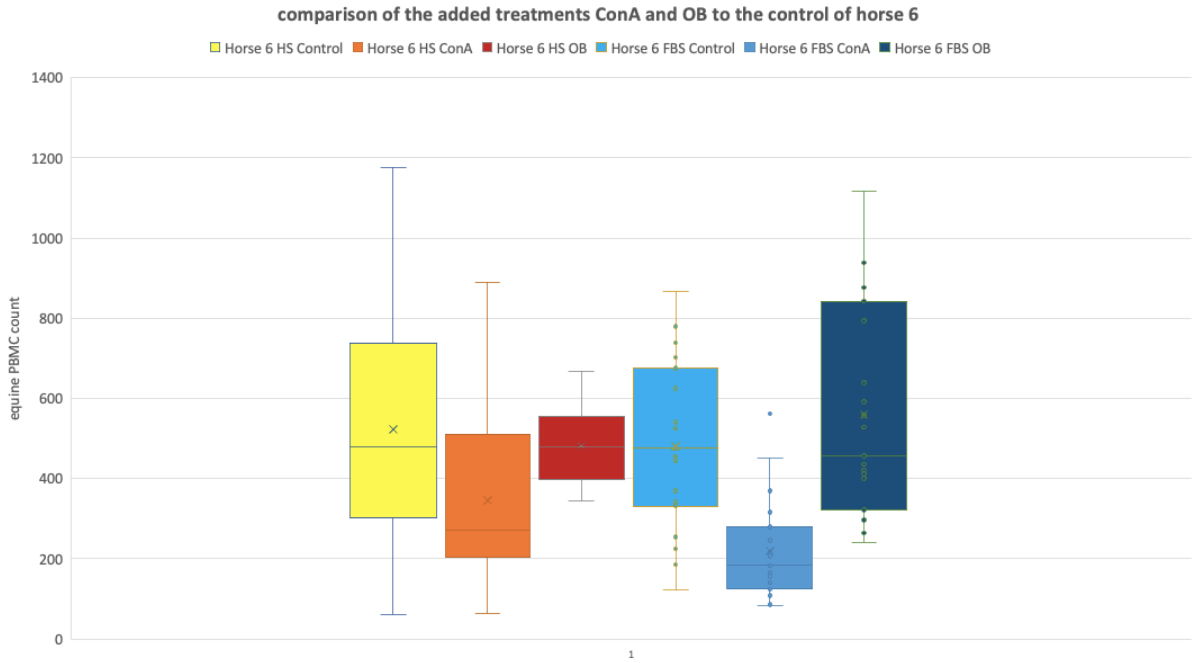

Figure 29: *comparison from horse 6 with the two different serum substitutes FBS and HS and different treatments with ConA and OB and the negative control*

The colored box blots assigned to the serum substitutes and additives used show the distribution of the cell count in comparison with the two different serum substitutes HS and FBS used as well as the different additives ConA and OB.

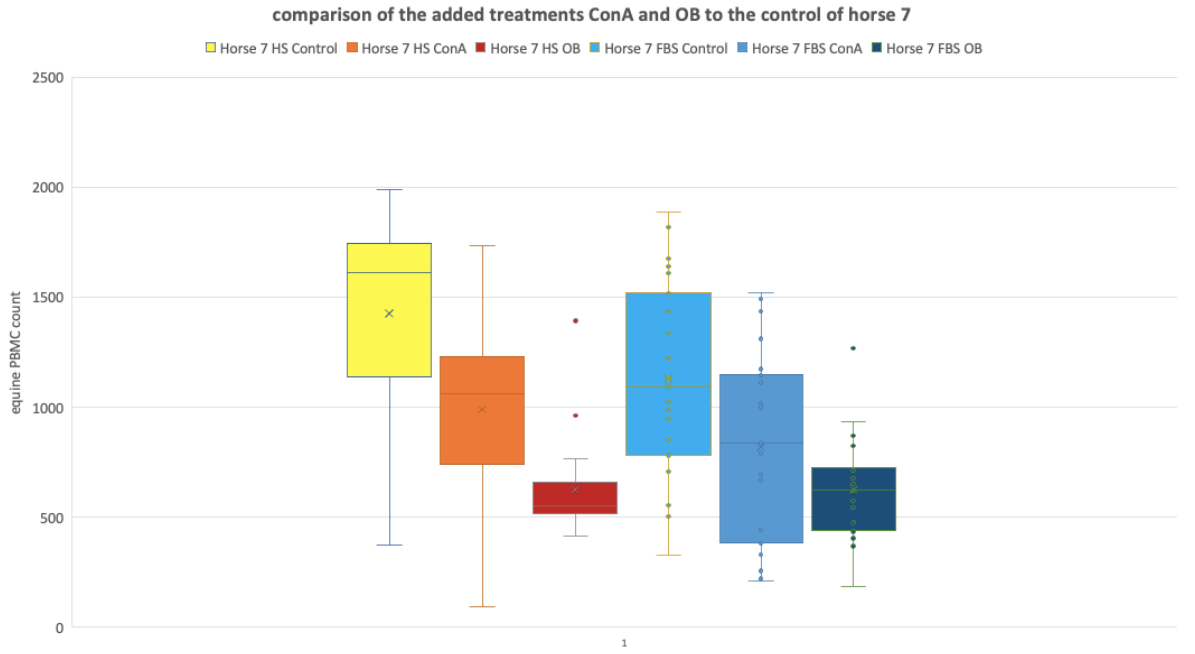

Figure 30: *comparison from horse 7 with the two different serum substitutes FBS and HS and different treatments with ConA and OB and the negative control*

The colored box blots assigned to the serum substitutes and additives used show the distribution of the cell count in comparison with the two different serum substitutes HS and FBS used as well as the different additives ConA and OB.

The images in figure 31 to 34 show the fluorescent antibodies of the different PBMCs that could be visualized by fluorescence in the “Incucyte S3” live cell imaging. Figure 31 shows the fluorescent CD4-Fitc antibodies, which appeared yellowish, after two days of incubation. In figure 32 you can see the fluorescent Mono-APC antibodies which appeared red after three days. Figures 33 and 34 include the yellowish appearing IL-17-FITC antibodies and the also yellow stained CD8-PE antibodies which were also clearly visible after two to three days in the incubator.

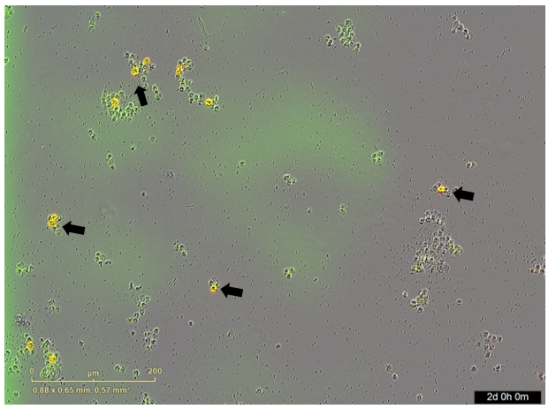

Figure 31: *Image of the fluorescent CD4-Fitc antibodies which have been stained yellow and are marked by the black arrows*

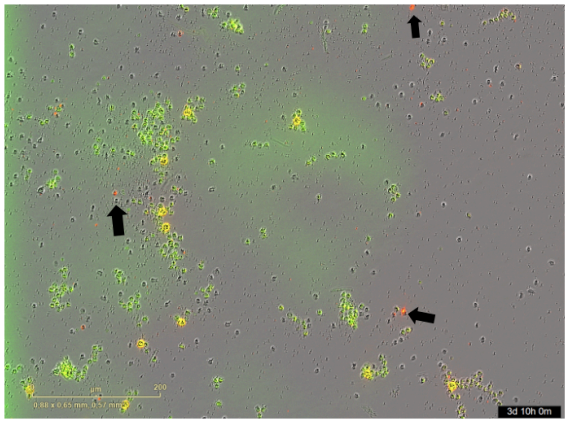

Figure 32: *Image of the fluorescent Mono-APC antibodies which have been stained red and are marked with the black arrows.*

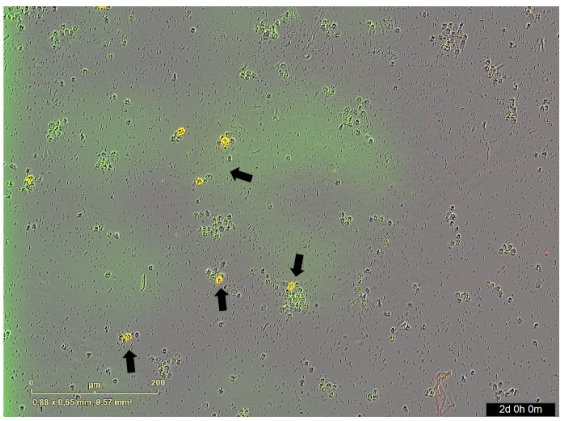

Figure 33: *Image of the fluorescent IL-17-FITC antibodies Which have been stained yellow and are marked by the black arrows.*

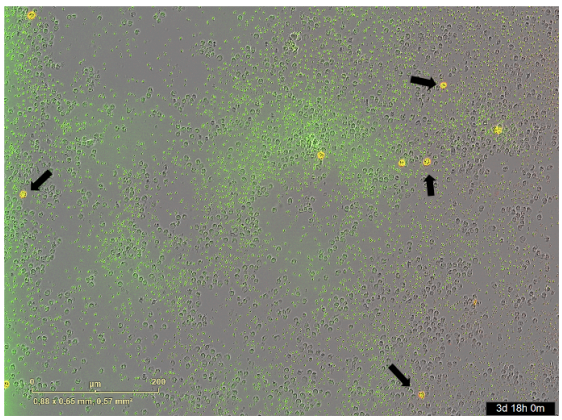

Figure 34: *Image of the fluorescent CD8-PE antibodies which have been stained yellow and are marked by the black arrows.*

Figures 35 to 47 show the analyzed data from the stained antibodies which were measured in the “BD Canto” flowcytometre. Each dot represents one cell and the cells inside the black circles and squares are the interesting ones concerning the experiment and were further analyzed. As a negative control RPMI medium with FBS and as a positive Control with ConA treatment were used. If one compares the results of the stained antibodies of CD3, CD4 and CD8, one can see, that the cell counts between the positive and the negative control hardly differ from each other (figure 35 to 40): with the CD3 AK, the cell count in the negative control with 88,2 was slightly higher than that of the positive control with 87,7. The CD4 antibodies behaved similarly. With the CD8 antibodies, the cell count of the positive control was higher with 3,6 than the one of the negative control with 3.59. The stained antibodies of the monocytes, B-cells and IL17 showed a slightly different picture (figure 41 to 46): for all three antibodies, the cell count of the positive control with the ConA was higher than the cell count of the negative control. Finally, figure 47 shows the cell count of the lymphocytes included in the treatment with RPMI medium, FBS as serum substitute and OmniBiotic.

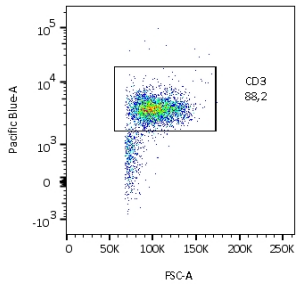

Figure 35: *Image of the stained CD3 antibodies in RPMI medium and FBS*

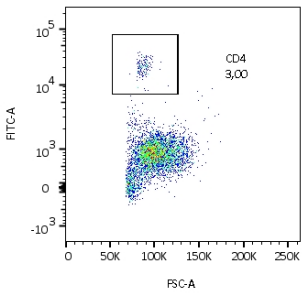

Figure 36: *Image of the stained CD4 antibodies in RPMI medium and FBS*

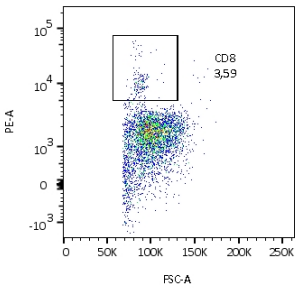

Figure 37: *Image of the stained CD8 antibodies in RPMI medium and FBS*

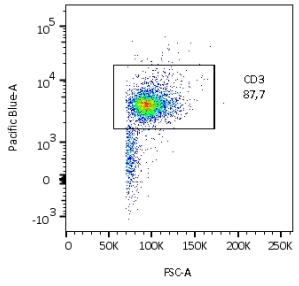

Figure 38: *Image of the stained CD3 antibodies in RPMI medium, FBS and ConA*

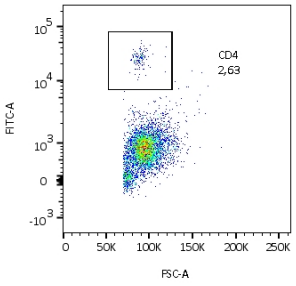

Figure 39: *image of the stained CD4 antibodies in RPMI medium, FBS and ConA*

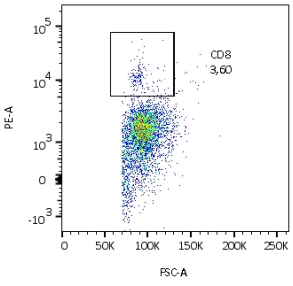

Figure 40: *Image of the stained CD8 antibodies in RPMI medium, FBS and ConA*

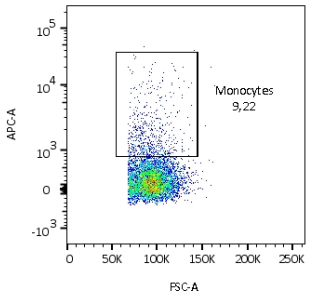

Figure 41: *Image of the stained monocytes antibodies in RPMI medium and FBS*

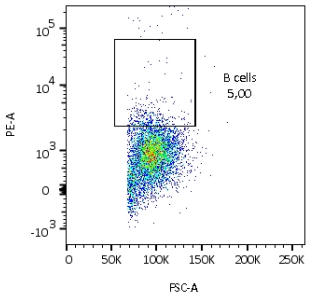

Figure 42: *Image of the stained B-cell antibodies in RPMI medium and FBS*

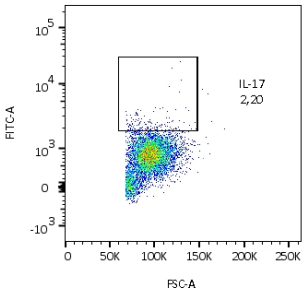

Figure 43: *Image of the stained IL-17 antibodies in RPMI medium and FBS*

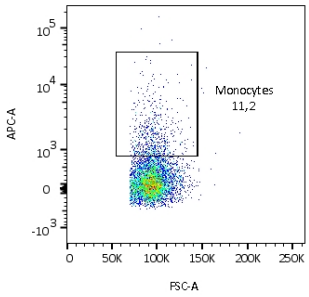

Figure 44: *Image of the stained monocytes antibodies in RPMI medium, FBS and ConA*

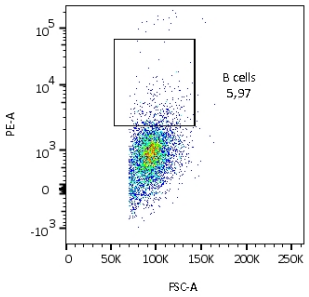

Figure 45: *Image of the stained B-cell antibodies in RPMI medium, FBS and ConA*

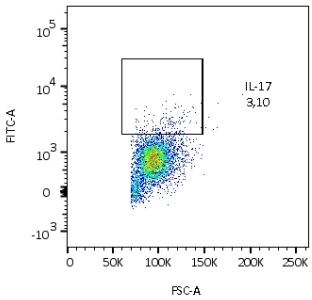

Figure 46: *Image of the stained IL-17 antibodies in RPMI medium, FBS and ConA*

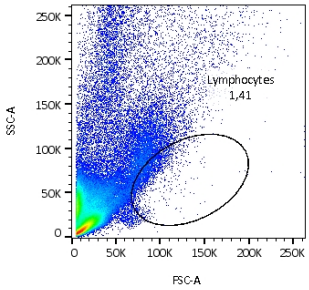

Figure 47: *Image of the stained lymphocytes in RPMI medium, FBS and OmniBiotic*

7. Discussion

Cell counts

Initially, the question arose as to why the cell counts varied so much between the different horses immediately after isolation, even though the same procedure was used to isolate the PBMCs using the same protocol (figure 2). The reason for this could be the different health conditions of the different horses, other underlying diseases as well as their age and immune status, about which, however, no further information were available in this trial. Hansen *et al.* and Horohov *et al.* confirm the assumption that the age of horses may have an influence on the amount of PBMCs, since a lower proliferation rate of these cells in connection with growth factors was detected in older patients *in vitro* (26) (27). Furthermore, errors in the processing may not be fully excluded, even if these were avoided.

Regarding the immense deviations of the cell counts of horse 6 and 7, an error during the measurement with incorrect focusing of the "Incucyte S3" was suspected, as the deflections were very regular and therefore could not be concluded to be due to cell development as the amount of cells couldn't decrease and then increase again in such short time intervals (figure 5).

Referring to the falling cell counts after 48h compared to the cell count after 24h in the incubator, regardless of treatment, it was concluded that possibly the nutrition factors from the serum substitutes as well as from the treatments were already depleted and the cells were therefore no longer in an optimal environment, which is why they decreased in number (figure 7). To circumvent this problem, the use of whole blood instead of PBMCs alone could be an alternative, as described in another study (28), as this would prevent the loss of important cells and endogenous substances and achieve better results. A technical problem regarding the incubator as well as the cell counter "Countess III" was rather unlikely, since no error message or similar were displayed, but cannot be ruled out entirely. The comparatively higher cell counts of horse 7 after 48h in the incubator were interpreted as possibly indicating that this horse had a better immune status than horse 6 and therefore the PBMCs showed a higher survival and even growth rate (figure 8).

ConcavalinA

Regarding the results representing the course of cell counts of horse 1 to 7 measured, it could be concluded that the positive control with ConA had no clear effect, as hardly any difference could be traced to the negative control (Figure 3 to 6). As Gunther *et al.* described, Con A can have an impact on the cell behavior but due to its variety, it can lead to interactions of different cell types, which then agglutinate with each other (22). This case could also have occurred with the equine PBMCs examined here, which is why the measured values of the positive control with the ConA do not differ significantly from those of the negative control without any treatment. Another study came to a similar conclusion regarding the influence of ConA on the growth of equine PBMCs, as it was also found that the expression of certain factors of the equine PBMCs was lower when treated with ConA alone than when another stimulating factor was added (29). Nevertheless, ConA is also used frequently in human medicine as a treatment to stimulate the growth of PBMCs (30); Here, just as in the experiment described in this study, PBMCs were isolated from blood and cultured with RPMI medium, FBS and ConA. However, the results of this pilot study indicate that in further studies an alternative positive control should be used to control for the capacity to respond.

Different media and serum substitutes in combination with OmniBiotic

The tendency for proliferation of PBMCs of horse 6 and 7 with OmniBiotic as treatment in the RPMI medium and FC1 as a serum substitute as well as the FBS serum suggested, that the probiotic with the right serum supplement, i.e., the right environmental conditions, may have a positive effect on the immune cells (figure 6). Surprisingly, the DMEM medium in combination with the HS as serum substitute visibly worsened the cell count in comparison, although the serum was individually derived from the respective horses. However, it is questionable whether this negative behavior of PBMCs was due to the DMEM medium or the HS serum substitute, because the equine PBMCs also behaved worse in figure 14 with HS and OB even without a specific medium than compared to figure 13 with FBS and OB. Similar results were also found in figure 15, 16 and 17. Nevertheless it cannot be excluded that negative behavior of equine PBMCs is due to the DMEM medium, as in figure 21 as a direct comparison of the two media, the cell count in the DMEM medium was also clearly lower than in the RPMI medium, just as in figure 22 with FC1 as a serum substitute and figure 23 with OB. A similar comparison of the

two different media was described in a study, where they tested the influence of the media on the proliferation of canine periosteum-derived cells (31); In this case, however, the cells felt more comfortable in the DMEM medium, but this may depend on the type of cells testes, which differ from PBMCs. Since the sera of the two horses were mixed/ combined, it was suspected, that perhaps the PBMCs of one horse did not correlate with the ingredients of the serum of the other horse and therefore a negative trend in the cell counts developed. In the "Comparative study of the effects of fetal bovine serum versus horse serum on growth and differentiation of primary equine blood bronchial fibroblasts" from Franke et at immense differences were also found between the two serum substitutes with regard to the growth and proliferation of the cells, whereby, unlike in this experiment, no equine PBMCs but equine blood bronchial fibroblasts were examined (32).

It was also evident, that the cell proliferation of the PBMCs was lowest with the OmniBiotic treatment compared to the negative and positive control. However, this result does not necessarily indicate a worse influence of the probiotic on the behavior of PBMCs but could also be due to the fact that the two bacterial strains of the probiotic also used the nutrition factors from the media and sera and thus fewer resources were available for the PBMCs themselves, which is why they could not proliferate to the extent that might have been possible under better circumstances. The study mentioned above (28) also included an attempt to stimulate proinflammatory factors by means of the probiotic bacterium *Enterococcus faecium* and concluded that the cell number of PBMCs in a suitable *in vitro* environment can develop positively through the influx of the probiotic bacterium.

The course of the cell numbers and the conclusion that the PBMCs increased only in combination with FBS as serum substitute and OB as treatment showed repeatedly that the probiotic did have a positive effect on immune cell survival and growth rate (Figure 9 to 14). Furthermore, those results underlined the results already obtained from figure 5 where the PBMCs also proliferated best in connection with the FBS serum substitute and the OmniBiotic treatment. The already known and described possible positive effect of probiotics on the immune system is known mainly via the gastrointestinal route, in which probiotics bind certain receptors in the intestine via which the immune system is activated or stimulated (33) (34). Positive immunomodulatory effects have been achieved not only in human medicine with the same bacterial strains used in this trial (*Enterococci* and *Lactobacilli*) (35) but also in veterinary

medicine; In addition to an increase in specific immune functions in young dogs through oral administration of the probiotic bacterium *Enterococcus faecium* (15) as well as *Lactobacillus acidophilus* (36), in vitro tests were also carried out in horses to determine how oral probiotics affect the gastrointestinal tract (37). In this trial, among others, the two already mentioned bacterial strains were used and it was shown that these probiotics could have a positive influence on the gastrointestinal tract of the horses, provided that their survival is ensured by enteric protection (37) because their main target is the large colon where most diseases are mainly located (5). The missing data mentioned above regarding the immunomodulatory effect of certain probiotic strains in horses (5) could potentially be supported by the results described in this study.

The comparison shows, in which serum substitute the PBMCs survive better, confirmed the previous statement as well, since the cell counts with FBS as serum substitute were always above those with HS, especially in the negative and positive control, with a few exceptions (figure 15 to 17). Nevertheless, it showed that with OmniBiotic as treatment, in horse 1, 1* and 5 the cell counts were higher with the horse serum. One reason for the better influence of the FBS serum substitute on the proliferation of the PBMCs could be that FBS originates from a juvenile animal and therefore harbors many growth factors. This presumably contributes to a better proliferation rate. Although the HS is the “natural” and individual serum substitute of each horse, but of an adult animal without juvenile growth factors.

In order to find out which serum substitute is best suited for the proliferation of PBMCs, the results provided that it may depend on the combination of the serum substitute and treatment and therefore an individual decision must be made which serum substitute is most suitable for which treatment (figure 18, 19 and 20). It could thus be seen, that ConA as a treatment acted best with the horses individual HS, while OmniBiotic with FC1 achieved the highest cell counts. The reason for this may be the more optimal ingredients of serum substitutes for the respective treatment.

It became clear, that the cells felt much more comfortable in RPMI medium than in the DMEM medium (figure 21, 22, 23). The higher cell numbers of PBMCs in the RPMI medium are probably due to its ingredients, which provide a better environment and nutrients for the cells than those of the DMEM medium.

It could be concluded that the majority of the PBMCs preferred the combination of FBS and OB, and generally tended to feel more comfortable in the FBS serum substitute than in the HS, which additionally underlines the cell preferences already identified above (Figure 24 to 30). The deviations that occurred could be mainly explained by individual differences regarding the horse's immune status or maybe a pipetting error.

Visualization of the stained antibodies

It was possible to visualize the stained antibodies of the immune cells CD3, CD4, CD8, Monocytes, B-Cells, and IL-17 with the "Incucyte S3" (figure 31 to 34). In other studies, the "Incucyte S3" has also been used to fluorescently visualize specific cell lines and even PBMCs as in a study from Lanigan TM (38). Although all antibodies could be visibly milled, they did not all show up exactly as expected. Especially the green fluorescence was rather yellowish, which led to the assumption that either the staining of the green antibodies was not intense enough, the green antibodies or their referring immune cells were not present in sufficient numbers or that there were technical problems with the presentation of the green fluorescence. In comparison the stained antibodies could be also visualized via the "BD Canto" flowcytometre (figure 35 to 47). The same method of visualizing antibodies of certain immune cells by means of immunofluorescence is not only used in human medicine (39) but also for a long time in veterinary medicine (40) and thus plays an important role in studies concerning the immune system (25). The barely detectable difference in the number of antibodies between the positive and negative control was consistent with the findings from the results described above, in which the cell counts were also predominantly the same for the positive and negative control. However, only two horses were analyzed by flow cytometry and therefore a general statement is not possible.

8. Conclusion

The probiotic product „OmniBiotic Cat & Dog" with its two bacterial strains from the institute ALLERGOSAN seems to have a direct immunomodulatory effect to the adaptive immune cells (PBMCs) in equine blood *in vitro* if all other influencing factors such as the medium, serum substitute and the growth conditions fit. The experiment described in this paper has shown that the RPMI medium was more suitable than the DMEM medium and FBS turned out to be the best of the three serum substitutes tested, next to the horse serum and the FC1, to support the PBMCs in the treatment with the probiotic product „OmniBiotic Cat & Dog" best. Nevertheless, there may be individual differences and deviations that must be reconsidered and, if necessary, adapted for each experiment. Lastly, it was clearly shown, that the stained antibodies of immune cells could be visualized in the live cell imaging of the "Incucyte S3" even though it is not completely clear if all fluorescent colors were visualized properly.

For more detailed knowledge and information especially about the immunomodulatory effect of the probiotic product „OmniBiotic Cat & Dog" to equine adaptive immune cells (PBMCs), further experiments should be conducted and *in vivo* studies on horses with the probiotic as an oral feed additive should be carried out in order to gain insights of possible positive effects.

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