The Effects of Myrtle Extract added to drinking Milk on Performance and Some Blood Parameters of Simmental Calves

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PhD Thesis

Supervisor: Prof. Dr. Ismail BAYRAM

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INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF ANIMAL NUTRITION & NUTRITIONAL DISEASES

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ÖZET

Tek sağlık kavramı, çevrenin, hayvanların ve insanların birbiriyle ilişkili ve birbirine bağımlı olduğunu gösteren nispeten yeni bir terimdir. Tek sağlık kavramının önemli yönlerinden biri hayvansal kaynaklardan gelen besindir. "Gıda güvenliği için yem güvenliği" kavramı, insan tüketimi için antibiyotik kalıntısı veya büyüme hormonu içermeyen güvenli ve sağlıklı gıdanın bulunmasını sağlamak önemlidir. Bunun için gıda üreten hayvanlara organik ve doğal yem veya doğal yem katkı maddelerinin verilmesi gerekmektedir. Sağlıklı bir yaşam sürmek için insanların, yalnızca sağlıklı gıda üreten hayvanlardan elde edilebilecek yeterli amino asit profiline sahip kaliteli hayvansal proteine ihtiyaçları vardır. Bu çalışmada, mersin bitkisi özütünün büyüme performansı, ishal oranı ve bağışıklık fonksiyonu üzerindeki etkileri, sütten kesilme öncesi Simental ırkı süt buzağılarında değerlendirildi. Bireysel olarak barındırılan 48 Simental buzağı (40 ± 4) doğum ağırlığı, 3 günlük yaşta), her grupta 12 buzağı (6 erkek, 6 dişi) olacak şekilde dört gruba rastgele atandı; C (kontrol grubu), MP (10 g probiotik/buzağı/gün), MM (50 mL mersin bitkisi özütü /buzağı/gün) ve MMP (10 g probiotik + 50 mL mersin bitkisi özütü /buzağı/gün) grupları. Deney periyodu 75 gündü. Tüm buzağılar deneyin sonuna kadar 2 × 2 L tam yağlı sütle (4 L/gün) beslenmiştir. Başlangıç yemi 2. haftadan ve kaba yem 5. haftadan sonra deney süresince farklı kovalarda ad libitum olarak sunulmuştur. Üçüncü haftanın başında (15. günden itibaren) yemler buzağılara her gün tartılarak taze olarak verildi. Ertesi gün kalan yemler yem kaplarından toplanarak her buzağıya özel, hava geçirmez boş torbalarda saklandı ve bu uygulama 7 gün boyunca devam etti. Sekizinci günde, son 7 günün yemi kalan çuvallar tartılarak yem tüketimleri belirlenmiştir. Yem tüketimi ve fekal skor puanlaması deneme sonuna kadar her gün kaydedildi. Sağlık durumu, vücut ısısı, dışkı kıvamı (ishalin incelenmesi için), ishal (kuru madde oranı %10'un altında olan dışkı), solunum (pnömoniyi kontrol etmek için), deri (herhangi bir ekto-parazit istilası olup olmadığını anlamak için), anhidroz, alopesi, herhangi bir mantar enfeksiyonu açısından incelendi. Olası hastalıkları kontrol etmek için buzağıların genel vücut durumu günlük olarak takip edildi. Deneme grubu buzağılarda şiddetli bir ishal görülmedi, ishal vakaları olduğunda da daha az sıklıkta ve şiddette görüldü. Bu nedenle tüm deneme süresi boyunca sıvı tedavisine ihtiyaç duyulmadı. Denemede ishalden etkilenen buzağılar, takviyeli veya takviyesiz gruptan bağımsız olarak, aynı antibiyotik tedavi prosedürüne göre tedavi edildi. Tedavilere art arda 3 gün devam edildi. Tüm buzağıların vücut ağırlığı ölçümü 3 kez yapılmıştır; doğumda, 1. aydaki ağırlıkta ve denemenin 75. günündeki son ağırlık. Kan örnekleri denemenin 7., 20., 40., 60. ve 75. günlerinde

alındı. Buzağıların tamamı 21 günlük olduklarında solunum sistemi hastalıklarından korumak amacıyla boyun bölgesinden subkutan olarak 5 ml Bovilis (Bovipast, MSD) ile aşılandı. Aşının rapel aşısı ilk uygulamadan 21 gün sonra (buzağılar 42 günlükken) aynı şekilde ve dozda yapıldı. Aşı, TCID50 değeri 105,5 olan inaktif BRS virüsü (EV 908), TCID50 değeri 105,5 olan inaktif Parainfluenza 3 virüsü (SF-4 Reisinger suşu) ve 9X109 inaktif Pasteurella (Mannheimia) haemolytica hücrelerini (serotip A1) içermektedir. Canlı ağırlık, yem tüketimi, yemden yararlanma oranları (FCR) gibi performans parametreleri ile kan biyokimyasal ve hematolojik parametrelerinin istatistik değerlendirilmesinde GLM metodu kullanılmıştır. Anlamlılık düzeyi ($p \leq 0,05$) olarak belirlendi. Araştırmada, MM grubundaki buzağıların ortlama yemi tüketimi diğer gruplardan daha yüksek ($p=0.0001$) bulundu. MM katkılı gruplarda (MM ve MMP grupları) ortalama canlı ağırlık, kontrol ve MP'ten daha yüksek ($p=0.0085$) bulundu. Buna karşın yemden yararlanma oranları bakımından gruplar arasında benzer bulundu. Deneme boyunca mersin bitkisi özütü ve probiyotik ilaveli buzağılarda ishal ve pnömoni görülme sıklığı kontrol grubuna kıyasla daha düşüktü. Araştırmada incelenen kan biyokimya parametrelerinden glikoz, NEFA, BHBA, BUN, ALT, GGT ve kreatinin düzeyleri bakımından gruplar arasında benzer saptandı. Buna karşın total protein ($p=0.0001$), AST ($p=0.0352$) ve IgG ($p=0.0001$) değerleri açısından gruplar arasında anlamlı farklılıklar bulunmuştur. Tüm deneme gruplarının IgG seviyesi kontrol grubuna kıyasla daha yüksektir. Kan fizyoloji parametrelerinden MCH ($p=0.0022$), MCHC ($p=0.0097$) ve WBC ($p=0.0001$) değerleri dışında diğer parametrelerde anlamlı farklılıklar bulunmamıştır. Böylece deneme süresince buzağılarda yem tüketimi, büyüme performansı ve bağışıklık, MM katkısından önemli ölçüde etkilenmiştir. Mersin bitkisi özütü, sütten kesim öncesi buzağılarda ishal görülme sıklığını azaltmıştır. Kontrol grubuyla karşılaştırıldığında büyüme performansında önemli bir iyileşme, buzağı ishalinde azalma ve serum bağışıklığında genel bir iyileşme vardı. Bu nedenle, sütten kesilmeden önceki dönemde buzağıların sütlerine tek başına mersin bitkisi özütü veya probiyotik ile karışımının eklenmesi önerilmektedir.

Anahtar kelemleri: Buzağı, Mersin bitkisi özütü, Performans, Probiyotik, Süt

ABSTRACT

The concept of one health is relatively a new term indicating that the environment, the animals and the humans are inter-dependent to each other. One of the important aspects of one health concept is food obtained from the animal sources. The concept of "feed safety for food safety" illustrates that the organic and natural feed or feed additives should be given to the food producing animals to ensure the availability of safe and healthy food, without any antibiotic residues or growth hormones, for the human consumption. To lead a healthy life, the humans need good quality animal protein having good profile of amino acids which can only be obtained from the healthy food producing animals. In this study, the effects of myrtle plant extract on growth performance, diarrhea rate and immune function were evaluated in Simmental dairy calves pre-weaning. Individually housed 48 Simmental calves (40 ± 4) birth weight, 3 days old) were randomly assigned to four groups with 12 calves (6 males, 6 females) in each group; C (control group), MP (10 g probiotic/calf/day), MM (50 mL myrtle extract/calf/day) and MMP (10 g probiotic + 50 mL myrtle extract/calf/day) groups. The experimental period was 75 days. All calves were fed 2 × 2 L whole milk (4 L/day) until the end of the experiment. Starter feed was offered ad libitum after 2 weeks and roughage after 4 weeks in different buckets throughout the experiment. In the beginning of 3rd week (from the 15th day), weighed and fresh feed was offered to the calves every day. On next day the remaining feed was collected from the feed pots and stored in air sealed empty bags specific for every calf and this practice kept on going for next 7 days. On the 8th day the bags, having remaining feed of previous 7 days, were weighed. Feed intake and fecal score were recorded every day before feeding until the end of the experiment. The health status i.e. temperature, fecal consistency (to examine diarrhea), scours (feces with dry matter below 10 %), respiration (to check pneumonia), skin was examined for any ecto-parasitic infestation, anhydrosis, alopecia, any fungal infection and overall body condition of calves was daily examined to check any possible disease. In the supplemented calves no severity, less frequency with no diarrhea sequels was observed so fluid therapy was not required during whole of the duration of trial. Calves affected by diarrhea in the trial were treated according to the same antibiotic treatment procedure, regardless of the supplemented or non-supplemented group. Treatments were continued for 3 consecutive days. The treatment was continued for 3 consecutive days. Body weight of all calves was measured for 3 times; at birth, weight at 1 month and final weight at day 75 of the trial. Blood samples were taken on the 7^{th}, 20^{th}, 40^{th}, 60^{th} and 75^{th} days of the trial. All calves were vaccinated

with 5 ml Bovilis (Bovipast, MSD) subcutaneously from the neck region to protect them from respiratory system diseases when they reached the age of 21 days. The booster of the vaccine was inoculated 21 days after the first administration (when the calves were 42 days old). The vaccine contains inactive BRS virus (EV 908) with a TCID50 value of 105.5, inactive Parainfluenza 3 virus with a TCID50 value of 105.5 (SF-4 Reisinger strain) and 9X109 inactive Pasteurella (Mannheimia) haemolytica cells (serotype A1). The growth parameters BW, FI, FCR, biochemical and hematological were statistically evaluated as GLM. Significance level was set at ($p \leq 0.05$). In the study, the feed intake of calves in the MM group was higher (p=0.0001) than the other groups. Body weight was higher (p=0.0085) in MM supplemented groups (MM and MMP groups) than control and MP groups. However, feed conversion ratio was similar between the groups. The incidence of frequency, severity and duration of diarrhae and pneumonia were lower in myrtle plant extract and probiotic supplemented calves compared to the control group. Glucose, NEFA, BHBA, BUN, ALT, GGT and creatinine levels of blood biochemistry parameters were similar between the groups. However, significant differences were found between the groups in terms of total protein (p=0.0001), AST (p=0.0352) and IgG (p=0.0001) levels. IgG levels were higher in all experimental groups compared to the control group. There were no significant differences in blood physiology parameters except MCH (p=0.0022), MCHC (p=0.0097) and WBC (p=0.0001) values. Thus, feed intake, growth performance and immunity of calves were significantly affected by MM supplementation during the experiment. MM decreased the incidence of diarrhea in calves before weaning. There was a significant improvement in growth performance, reduction in calf diarrhea and an overall improvement in serum immunity compared to the control group. Therefore, we recommend supplementing the milk of dairy calves with myrtle extract alone or mixture of myrtle extract and probiotics during the pre-weaning period.

Key words: Calves, Milk, Mrytle extract, Performance, Probiotic,

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ABDUL QUDOOS

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ABBREVIATIONS

ADWG Average daily weight gain
BHBA beta hydroxybutric acid
BW Body weight
BWG Body weight gain
CH_4 Methane
EDTA Ethylenediamine tetra acetic acid
EO Essential oil
FE Feed efficiency
FMD Foot and mouth disease
GI Gastrointestinal
GIT Gastrointestinal tract
GRAS Generally regarded as safe
Hb Hemoglobin
HCT Hematocrit
IgA Immunoglobulin A
IgG Immunoglobulin G
IgM Immunoglobulin M
LAB Lacto acidic bacteria
MCHC Mean corpuscular hemoglobin concentration
MCV Mean corpuscular volume
MCH Mean corpuscular hemoglobin
MEO Myrtle essential oil
MPE Myrtle plant extract
MIC Minimum inhibitory concentration
MM Milk and myrtle
MP Milk and probiotic
NEFA Nonesterified fatty acids
NO Nitric oxide
PFA Phytogenic feed additive
PLT Platelets
RBC Red blood cells
RDW Red cell distribution
USFA Unsaturated fatty acid
VFA Volatile fatty acid
WBC White blood cell
WHO World health organization

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1. INTRODUCTION

The people in the ancient time used to be benefited from the plants in several ways such as food, clothing, shelter, and most importantly as folk medicine or ethno-medicine. It is very interesting to know that even today most of the modern day medicines e.g. morphine, codeine, quinine, and atropine, all are derived from plants. The phytomedicines are a much better option because these are organic in nature, have no untoward or side effects, no detrimental effects if used within prescribed dose. The phytomedicines can also be used as nutraceuticals (food items which are used to treat the diseases) (Bjelakovic, et al., 2014). In USA almost 25% of the prescribed drugs are derived from plants. The WHO estimates that throughout the world approximately 4 billion people use herbal medicine. Keeping in view the importance of phytomedicine, the pharmaceutical companies execute scientific research trials on plants. The phytomedicines are composed of chemicals which establish the optimum response either by improving the cell-mediated immunity, or by activation of antigen-specific cytotoxic T-lymphocytes, or by releasing some cytokines (Saxena, et al., 2013). The herb and plant extracts are mostly used due to the fact that these are cheap, easily available, and have broad spectrum effectivity against pathogens (Dhama, et al., 2018). The plant extracts can be supplemented through different ways but the per-oral route is considered as the most effective way of supplementation (Jeney, et al., 2015). It is suggested to use the natural form of the plants so that the benefit could be taken from all the phytobioactive compounds.

1.1 Calf Management at Dairy Farms

At the time of birth, the calves are agammaglobulenemic (they don't have natural immunity at the time of birth) and it is highly recommended to give enough quantity 10% of body weight and quality (at least 50 g IgG/liter of colostrum) to the newly born calves as quickly as possible after the birth of calf. The colostrum has antibodies or immunoglobulins (immunoglobulin G IgG, immunoglobulin A IgA, and immunoglobulin M IgM), for calves to transfer the passive immunity. If by somehow, within an hour after birth of the calf, enough quality as well as quantity of colostrum could not be given to calf then calf becomes immunologically and physically vulnerable. In most of countries newly born calves of ruminants either do not get or deprived of their basic

right to get required quantity and quality of colostrum and hence do not get passive immunity from their dams which makes them susceptible to the calf-hood diseases which leads to calf mortality almost 7.8% and economic losses (NAHMS-USDA, 2007).

It is pertinent to mention that the initial few weeks of calf are very crucial because the gastrointestinal infection in the initial few weeks can be detrimental and lead to delayed growth in the coming days which badly affect the productivity, so therefore it is very important to reduce the chances of infection in first few weeks of life of calf (Rosmini, et al., 2004). The stocking density of calves in small and confined area (intensive rearing system) can also increase the chances of gastrointestinal GI infections which can be minimized by evenly distributing and housing the calves in their respective calf hutches in calf shelter (Callaway, et al., 2002).

1.2 Neonatal Calf Diarrhea

It is considered as one of crucial causes of financial losses of dairy and beef industry globally, which results in growth and production related problems, increased treatment cost, and/or eventually the calf dies (USDA, 2018). Throughout the world the bovine calves, generally in pre-weaning phase and particularly in the 1st month of their life, are vulnerable and mostly affected by enteric diseases i.e. diarrhea, whereas morbidity (56%) and mortality (32%) is reported (Urie, et al., 2018).

Diarrhea is a multifactorial disease caused by different pathogenic microbes, the contaminated surrounding environment, cold temperature, and immune status of the calf (De La Fuente, et al., 1998). The increased incidence rate of diarrhea in neonatal calves, besides having long-lasting economic effects, has severe impacts on health and welfare of the calves (Bartels, et al., 2010), it also affects other important parameters e.g. reduced average daily weight gain ADWG by 50 g/day (Anderson, et al., 2003), requires 3 times more artificial inseminations to get first pregnancy and first calving after 30th month of life, and a decreased yield of more than 300 kg milk in the first lactation (Abuelo, et al., 2021). The diarrhea in neonatal calves can economically be detrimental (costs on an average 56 $ and 5.1% mortality ratio) to the dairy farmers in the form of treatment and labor costs (Windeyer, et al., 2014).

1.7 Probiotics

According to revised definition by Food and Agriculture Organization FAO/WHO, the probiotics are "live microbes, which, when supplemented in sufficient quantities, impart a healthy effect on host" (Fuller, 1989). In this regard lactic acid bacteria which belong to *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*, are recognized as beneficial to host and are used as probiotics. The probiotics have the capability to establish a healthy environment of intestine by stimulating the propagation and colonization of beneficial microbiota, restricting the gut pathogenic bacteria from colonizing, improving the digestion by either lowering or adjusting the pH, and enhancing the mucosal immunity. The supplemented probiotics should not disturb the local microbial population already residing the gut and should work synergistically for the betterment of the host. Although normally the LAB are present in GIT of calves but by adopting the probiotic strategy (increasing the quantity of LAB in intestine from external sources) the colonization of LAB can be promoted which can dismantle and neutralize the pathogenic bacteria in the GIT of calves by stimulating the immune response (Frizzo, et al., 2010).

The probiotic preparations are composed of either one or more than one microbial strain and it is supplemented to animals in the form of granules, powder, paste or tablets. The microbes, used in manufacturing of the probiotic preparations, are obtained from the gut of the same animals for whom the probiotic preparations are being manufactured so that, when supplemented to the same species of animals, the microbial material can adapt to the similar gut environment (Mizak, et al. 2012). The probiotic can be supplemented to all the food producing animals which are in their different life stages but it's use in newly born ruminant calves is very fruitful because the gut of newly born calves is not very much established and its use can expedite and accelerate the gut as well as rumen development. The use of probiotic in beef calves in their pre-weaning phase can give a good start to the calf which can lead to effective nutrient utilization in gut which in turn leads to an increased growth production in pre-weaning as well as in post-weaning phase. In dairy cows the probiotics have the potential to improve milkiness through effective utilization of nutrients by enriching gut and rumen microbiota (Semeniuk, et al., 2008).

1.3.1 Types of Probiotics

Among all of the probiotics, LAB are most commonly known probiotic and these belong to *Lactobacillus spp.* and *Bifidobacterium spp.* Some other microbes include *Saccharomyces*

cerevisiae, *Lactococcus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Bacillus cereus*, etc. The *Lactobacillus* and *Bifidobacterium* are among the Generally Recognized as Safe GRAS. The *Lactobacillus spp.* and *Bifidobacterium spp.* are isolated from small and large intestine, respectively (Homayouni, 2012).

1.4 Effects of Direct Fed Microbial on Young Calves' performance

Similar to that in human beings, the animals also have a complex microbiome in the digestive tract and by adopting certain nutritional techniques, this microbiome can be potentiated not only to perform optimum production performance but also to restore the health status of the animals. The optimum production from the animals can be obtained by potentiating the beneficial bacteria to perform up to their maximum capacity and by killing the pathogenic bacteria residing in the digestive tract. The approach of killing pathogenic bacteria by administrating antibiotics is not justifiable because of the untoward effects of antibiotics on the GIT microbiota and also due to antibiotic residues deposited in the edible food products of food producing animals (Tang, et al., 2017). The other approach is the use of live microorganisms (probiotics), which impart healthy effects on beneficial gastrointestinal microbiota of the animals when administered in adequate amounts. The usage of probiotics as feed additive in the diet of dairy animals not only establishes healthy environment in GIT, potentiates the beneficial bacteria to perform up to their optimum capacity, but also there is no risk to health of human beings (Hammon, et al., 2020). The lactic acid bacteria are proved to be a tool to potentiate the beneficial bacteria and to prevent the establishment of pathogenic bacteria (Signorini, et al., 2012). Additionally, the *Bacillus spp.* reduce the colonies of pathogenic bacteria by improving the immunity level of epithelial cells (Piewngam, et al., 2018).

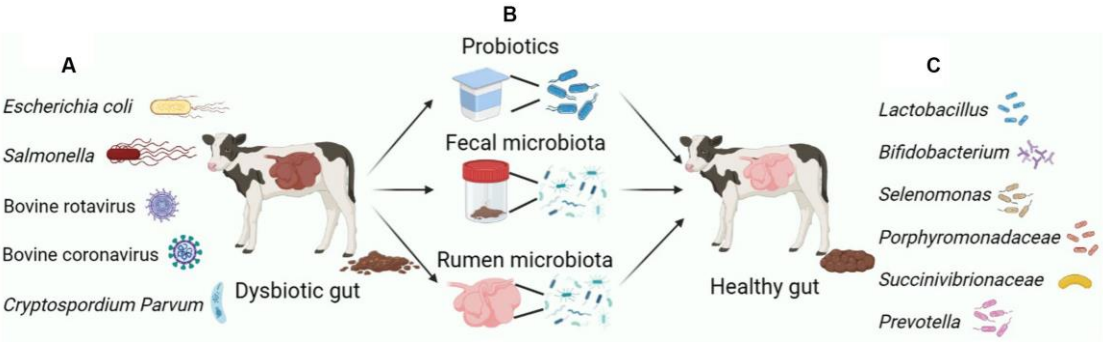


Figure 1.1: Important microbiota and their respective effects on gut health

The weaning is very important event of the life of a calf where it is gradually shifted from liquid diet to solid diet, this change makes the calf susceptible to disease and subjects it to the environmental stress. Although this change is accompanied by stress and disease but it is pivotal for the calf to be shifted on solid diet as soon as possible. The weaning leads to rumen development and establishes ruminal microbiota which affect the productive performance and health of adult ruminants (Soberon and van Amburgh, 2013). Newly born calves always undergo stress due to transport, dehorning and vaccination (Krehbiel, et al., 2003). In the intensified system of dairy farms after birth the calves are separated from their dams which always become stressful for calves as they suffer from psychological disturbance, digestive problems and weight loss. At this time supplementing large amounts of probiotics can positively modulate the intestinal environment and bring the calves out of stressful conditions (Kung, Jr. 2001). Abe et al. (1995) reported that oral supplementation of beneficial microbes significantly (p<0.05) improved BWG, feed utilization, and less severe diarrhea in calves.

1.5 Use of Phytogenic Feed Additives in Dairy Animals

In the diets of food producing animals the phytogenic feed additives PFAs or plant-based substances are being extensively used for the last few years. There are different commercial products available in market composed of various plant-based feed additives. These products are either available in single formulation or the product composed of multiple plant substances. The plant-based materials which are used as feed additives are spices, non-volatile extracts, EO and herbs which are derived from anise, clove, thyme, melissa, fennel and many others as shown in table 1 (Mathe, 2007). The phytogenic feed additives are either fed as powder, granulated, or in liquid forms mixed in pre-mixture or feed. There is a technique of encapsulating the feed additive in order to protect the active ingredients of plant, to minimize the odor, to delay or to effectively release in digestive tract. On the other hand, the liquid PFA is supplemented either in drinking water, in milk replacer or these can also be sprayed on processed feed, such as pellet or extruded diets. In order to formulate a PFA, the flavoring characteristics and sufficient knowledge of plant is required. It is supposed that all the ingredients of a PFA act synergistically to make it a well-formulated, efficient and effective PFA.

Table 1.1: Herbs and their respective utilizable parts

Common name	Scientific name	Utilizable parts
Cinnamon	*Cinnamomum verum*	Bark
Citrus	*Citrus spp.*	Peel
Garlic	*Allium sativum*	Bulb
Ginger	*Zingiber officinale*	Rhizome
Onion	*Allium cepa*	Bulbs
Oregano	*Origanum vulgare*	Leaves
Peppermint	*Mentha piperita*	Leaves

The objective of using PFA in the diet of dairy animals is to improve feed intake, increase in saliva production, improve or manipulate ruminal fermentation to decrease CH_4 gas production, improve the gut environment, strengthen the intestinal microbiota to work efficiently, improve nutrient digestion, effective nutrient absorption and utilization, improve health status of animals, increase the production performance of animals, improve the quality of products obtained from animals and to overall improve the health and wellbeing of food producing animals (Karásková, et al., 2015; Upadhaya and Kim, 2017). Due to serious public health concerns, the synthetic antibiotics are banned in the diet of livestock animals, and their replacement with other natural, organic feed alternatives is pivotal and inevitable (Surai, 2014). Therefore, alternatives dietary feedstuffs including plant secondary metabolites, called as phytogenic feed additives, phytobiotics are proposed. In the entire world the use of herbs as nutraceuticals in animal diet has had an important and traditional way to treat diseases (Kumar, et al., 2014).

1.5.1 How Phytobioactive compounds work

Generally, the PFA works as antimicrobial, anti-inflammatory, antihypertensive, analgesic, diuretic, anthelmintic, gastroprotective, antidiabetic, renal and antioxidant, immunomodulatory, etc. The exact action mechanism of PFA is not understood up till now however its mechanism of action is associated with the chemical nature of compound. Furthermore, the feed of animal is supplemented with PFA, beneficial effects are exerted on intestinal microbes, toxic metabolites are decreased, and intestinal environment is improved which leads to improved performance of

animal (Kim, et al., 2015). The phytobioactive compounds also act by increasing the antioxidant status which improves performance of animals (Settle, et al., 2014).

The possible effects of PFAs inside the animal body are illustrated in Fig. 3 that include the digestive secretions (juices and enzymes), an alteration of immunological parameters, improved utilization of nutrients, better digestion, increased animal performance. The positive impacts of PFAs on intestinal system are manifested by better, quick and more nutrients uptake, better digestibility and an improved performance of animals. Furthermore, this provides a stable and smooth environment in the gut which facilitates the beneficial microbiota to work efficiently.

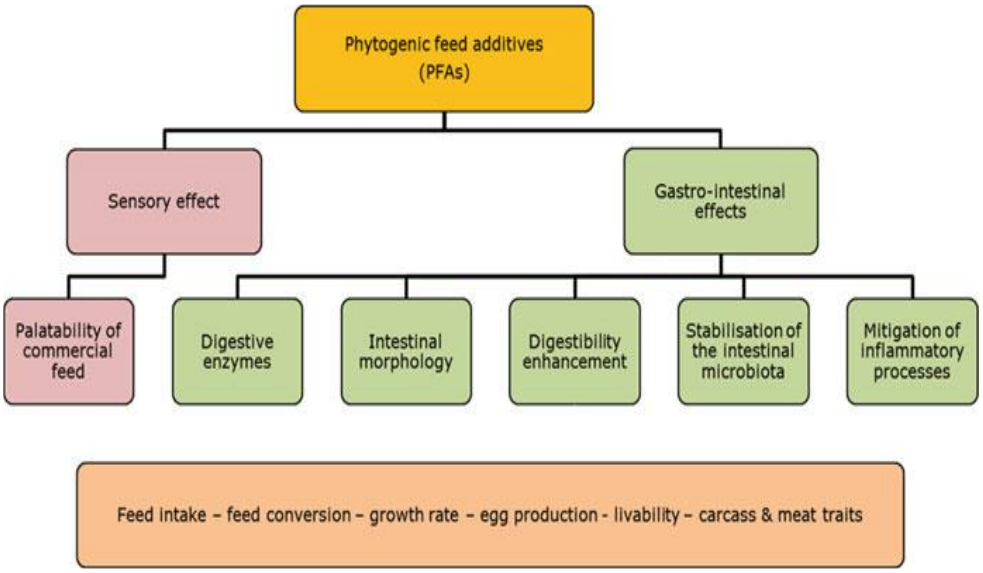


Figure 1.2: Possible effects of phytogenic feed additives

Regarding the general mode of action, there are 4 mode of action of PFAs are shown in Fig. 4 are mentioned below (Surai, 2014).

- By improving feed intake of animals
- By improving the fermentation of rumen
- By improving the absorption and digestion of nutrients
- By initiating direct and indirect anabolic activity.

Mode of action of PFA generally and largely depends on its specific structure, dosage, pharmacokinetics, pharmacodynamics, species and phase of animals, and the route of supplementation. The PFA contains various biological activities which are responsible for growth promotion and improving the production performance parameters of food producing animals but the exact mode of action of PFA is still not clearly elucidated.

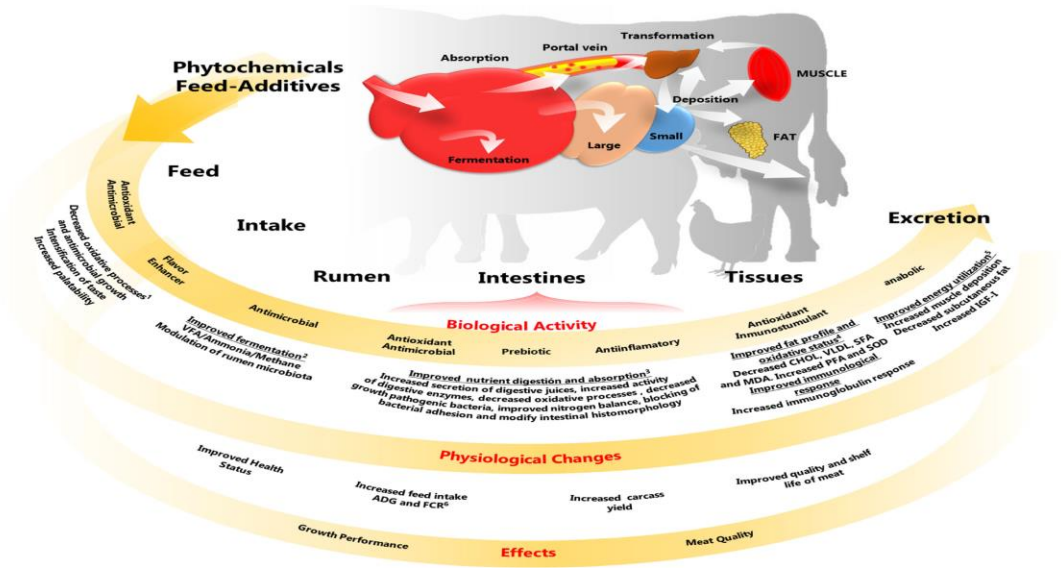


Figure 1.3: Schematic diagram of mechanism of action and effects of PFA

1.6 Effects of Phytogenic Feed Additive

1.6.1 Effects on Digestion

The term digestion or digestibility is defined as the limit to which the nutrients of feed to be digested and absorbed through the digestive tract of animals. The effective digestion of nutrients leads to better feed efficiency whereas less or poor digestibility leads to sub-optimal feed efficiency, less nutrient absorption through gut and less production performance by the animals. As a result of poor digestibility the feed is accumulated in digestive tract which leads to fermentation in large intestine by microbes and some undesired metabolites (ammonia, biogenic amines, etc.) are produced (Kroismayr, et al., 2008) whereas an enhanced inflammatory process is started due to the production of these undesired metabolites and resultantly normal physiology is changed which leads to decreased production of animals. In a trial the improved nutrients digestibility was found when the diet of weaning piglets was supplemented with PFA such as citrus essential oil, anise and oregano (Zitterl-Eglseer, et al., 2008). In a trial enhanced digestibility of nitrogen was reported when the diet of pigs was supplemented with mixture of cinnamon, essential oils, thyme, clove and oregano (Huang, et al., 2010).

1.6.2 Effects on Ruminal Fermentation

The rumen physiology can be altered by providing conducive environment to the ruminal microbes and in this way the working efficiency of ruminal microbiota is enhanced and consequently the nutritive parameters, overall health status and production performance of animals is improved. The ruminal microbes are involved in important processes of digestion of protein, biosynthesis of protein, digestion of carbohydrate and synthesis of vitamin which leads to biosynthesis of volatile fatty acids VFA (acetic acid, butyric acid and propionic acid), and among these VFAs the major proportion is of propionic acid because most of the energy maintenance and growth performance is related to this fatty acid FA (Choudhury, et al., 2015).

There are four below mentioned modes of action and by using which the phytochemical feed additives influence and bring alterations in ruminal microbes.

- Inhibition of synthesis of cell wall
- Disintegration of structure of cell wall
- Inhibition of synthesis of nucleic acid
- Inhibition of biosynthesis of protein

The PFA can bring alterations in microbiota that can affect the ruminal fermentation by reducing the methanogenesis and by increasing the VFA production especially the propionates and consequently feed utilization and ADWG can be improved (Cardozo, et al., 2006). A research trial was performed by (Dong, et al., 2010) wherein the feed of goat was supplemented with lucerne extract and a mixture of herbs and it resulted in reduced CH_4 gas production, increased VFA production particularly the propionate and decreased concentration of protozoa. In a trial it was observed that the effects of PFA supplementation to modulate the ruminal microbes was only observed in *in-vitro* research trials by using an isolated bacterial culture whereas no such ruminal microbial alteration and its beneficial effects were observed in *in-vivo* research trials with no positive effects on animal performance (Khiaosa-Ard and Zebeli, 2013). The increased dietary inclusion level prevented the microbial growth and affected the ruminal fermentation (Wanapat, et al., 2013).

1.6.3 Effects on Intestinal Microbiota

Any dietary or environmental change directly affects the working efficiency of the intestinal microbiome and due to this change the nutrient digestibility, nutrient absorption, feed conversion,

feed efficiency FE, health and production performance and the overall growth and development of animal is affected. Helander et al., (1998) documented that due to lipophilic nature the active substances of phytobioactive compounds enter the cell wherein they enter mitochondria, interrupts metabolism of energy and electron flow, which ultimately collapses the proton pump and drainage of ATP. In a trial (Mountzouris, et al., 2011) reported an effective change of caecal microbes when the diet of broiler poultry birds was supplemented with PFA @ 125 and 250 mg/kg. In the same trial an increasing supplementation of PFA led to an increased concentration of *Bifidobacterium, Lactobacillus,* etc. in poultry birds and lower concentration of caecal *coliforms* was also reported. It is supposed that the space emptied after the gradual reduction of unwanted or pathogenic bacteria i.e. *clostridia, coliforms, staphylococci,* etc. is filled by the beneficial bacteria *Lactobacillus* spp. after their proliferation and it is also supposed that *lactobacilli* spp. prevent pathogenic bacteria to colonize in gut (McReynolds, et al., 2009).

1.7 Myrtle plant (*Myrtus communis* Linn)

Myrtle (*Myrtus communis*) plant ever-green, flowering, spontaneous woody plant belongs to Mirtaceae family, subfamily Myrtoideae and this family is composed of almost 145 genera and 5500 species which are mostly found in the Mediterranean region. Apart from its native region, nearly 15 genera and 450 species are present in other regions e.g. Northeast Australia, New Zealand, Southeast Asia, Europe, Saharan mountains, Iran and Afghanistan. Majority of the plants are being utilized in animal diets (Smeti, et al., 2018) however the extract obtained from different parts of myrtle plant is not commonly used in ruminant nutrition (Tibaoui, et al., 2020).

1.7.1 Plant characteristics

The myrtus is an ever-green, aromatic, medicinally important, sclerophyll shrub and normally 3-7 m long. The plant has stiff branches & reddish twigs and the round berry (fruit, contains seeds) are reddish-blue to violet in color (Charles, 2013). The stem is branched and the dark green colored leaves are glossy, coriaceous, glabrous, paired or whorled, opposite, aromatic completely margined, ovate to lanceolate with stiff structure, acuminate having length of 2.5-3.8 cm and the lamina does not contain any glands. The plant has an axillary white or pinkish color, contains sweet

fragrant smell, star-like appearance, medium sized flowers having diameter of about 2 cm and has yellow colored anther. The petals of the flower are pure white colored and somewhat tomentose margin are covered with fine hairs (Sumbul, et al., 2011; Charles, 2013). The myrtle fruit is a multi-seeded (white or blue-black colored seeds) berry, ovoid-ellipsoid or orbicular, spherical pea shaped, and on an average 0.7-1.2 cm long. Initially the unripe berries (bitter in taste) are pale green colored, later on become deep red and on attaining maturity (sweet in taste) these become dark indigo colored (Figure 5). The berries grow in shades and bloom in summers (Sumbul, et al., 2011).

The plant contains some important phytobioactive chemicals compounds i.e. tannins, flavonoids, and EO (Baytop, 1999). The berries of myrtle contain polyphenols (Barboni, et al., 2010) which have important characteristics of anti-inflammatory (Rossi, et al., 2009), antibiotic and antioxidant (Tuberoso, et al., 2010). The polyphenols are important bioactive phytogenic compounds which exert several beneficial effects when these are incorporated in the diet of animals. The productive effects are due to unique chemical structure, inclusion level in the animal diet, and probably due to their synergistic effects either with the feed ingredients or with other phytochemicals.

Figure 1.4: *Myrtus communis,* leaves and berries

1.7.2 Distribution

The myrtle is evergreen, spontaneous and mostly grown in north western to eastern Mediterranean region, including the adjacent countries and the region of western Asia (Baytop, 1997). The plant grows in South American countries, northwestern Himalaya and in Australia also. In north western parts of India, the myrtle is cultivated in the form of gardens (Nadkarni, 1989). In Italy the myrtle

grows along the coasts, on the mountains and abundantly found on the islands (Cannas, et al., 2013).

In Portugal it is mostly found in south and central regions. In Tunisia only one specie *Myrtus communis* L. is found in coastal regions, on mountains, in northern parts of country. In the old Tunisian floral catalogue, two myrtle varieties (*Myrtus communis var. baetica* L and *Myrtus communis var. italica* L.) have same vegetative properties but with different fruit and leave size. The myrtle plant instinctively grows in France, Iran, Montenegro, Turkey, Greece, Morocco, Algeria, Croatia and Spain (Berka-Zougali, et al., 2012).

1.7.3 Traditional Application

Here in Turkey the leaves and fruits of myrtus are traditionally utilized for antisepsis particularly by the people living in rural areas (Baytop, 1999). In Italian ethno-medical practices, its fruit is used as first aid to dysentery and diarrhea (Gortzi, et al., 2008) whereas leaves and young branches are used for making an infusion which is effective as antiseptic, anti-inflammatory, stimulant, hypoglycemic agent and astringent. The infusion is used to cure eczema, asthma, diarrhea, psoriasis, urinary infections and gastrointestinal disorders, whereas its decoction is made from fruits and it is used as anti-diarrheal, anti-hemorrhoidal, etc (Ziyyat, et al., 1997).

1.7.4 Chemical Composition

Polyphenols are EOs, very important secondary metabolites of *Myrtus communis* and it also contains some important and specific chemical compounds such as terpineole, terpinolene, 1,8-cineole (13.5-19.6%), linalyl acetate (2.5-6%), tannins, linalool (7.7-15.8%), and flavonoids (Gardeli, et al., 2008). The leaf and flowers are chemically composed of EOs, phenolic acids, 1,8-cineole (~12-34%), tannins, α-pinene (~10-60%) and falvonoids (Wannes, et al., 2010) whereas berries contain fatty acids, anthocyanins (0.2-54%) and organic acids (9-52%), (Messaoud, et al., 2012).

Due to relative safety levels, multipurpose potential and greater acceptance by the consumers, the EOs and their components are considered important biological agents (Ormancey, et al., 2001). The EOs are present in glands, hairs, resins or cavities, ducts (Liolios, et al., 2010). Despite of their

crucial role in plant physiology, the EOs' hardly exceeds to 1% in their total concentration relative to the total plant mass (Bowles, 2003). Wannes, et al., (2010) the percentage of EO in leaf, flower and stem of myrtle plant are 0.61%, 0.30% and 0.08% (w/w), respectively. Physical properties of EOs include pale yellow or colorless, non-polar or weakly polar, hydrophobic, oils, waxes, soluble in alcohol and slightly soluble in water and these have less density as compared to that of water (Gupta, et al., 2010).

The chemical composition includes tricyclene, Z-3-hexenol, hexanol, α-pinene, δ-3-carene, E-2-hexenal, E-β-ocimene, geranyl 2-methylbutyrate, methyl eugenol, sabinene, geranyl acetate, E-oxide, p-cymene, geraniol, α-thujene, p-cymene-8-ol, β-pinene, myrtenyl acetate, α-terpineol, neryl acetate, 1,8-cineole, myrcene, tridecane, α-terpinene, cis-carveol, bornyl acetate, limonene, linalool, terpinolene, terpinene-4-ol, borneol, caryophyllene oxide, myrtenol, α-humulene, nerol, germacrene-D, linalyl acetate, eugenol, nonadecane, α-terpinyl acetate, α-phellandrene, β-caryophyllene, alloaromadendrene, thiophene, spathulenol, β-elemene, γ-terpinene, and trans-linalool oxide. There are 3 major categories which encompass all above mentioned compounds (Messaoud, et al., 2012; Aidi Wannes, et al., 2007 & Kafkas, et al., 2012).

- Phenylpropanoids
- Terpenoids (oxygenated sesquiterpenes and oxygenated monoterpenes)
- Terpenes (sesquiterpene hydrocarbons and monoterpene hydrocarbons)

Fragrance and chemical composition of EOs extracted from myrtus depend on environmental factors, growing conditions (nutrients concentration, humidity, temperature, day length, soil type, altitude, climate, available water, etc.), and vegetative phase e.g. prior to flowering (Messaoud, et al., 2012). The myrtle plant extracts contain below mentioned compounds.

- Phenolic acids i.e. ellagic, vanillic, garlic, ferulic acid syringic, and caffeic.
- Flavonoids i.e. myricetin, catechin, quercetin, and respective derivatives.
- Tannins i.e. hydrolysable tannins (gallotannins) and condensed tannins (proanthocianidins).

Some derivatives of myricetin and quercetin include myricetin-3-d-rahmnoside, myricetin-3-d-galactoside, catechin, quercetin-3-d-rahmnoside, and quercetin-3-rutinoside (Wannes, et al., 2010; Tuberoso, et al. 2010). The leaves of myrtus contain hydrolysable tannins (oenothein B, tellimagrandins-I, eugeniflorin D2, and tellimagrandins-II), polyphenolic chemical compounds (quinic acid 3,5-di-O-gallate and gallic acid), and flavonols are (myricetin 3-O-β-d galactoside,

myricetin 3-O-α-l-rhamnoside, myricetin 3-O-β-d-galactoside 6-O-gallate, and myricetin3-O-β-d-xyloside) (Yoshimura, et al., 2008).

1.7.5 Nutritional Composition

Nutritional composition of myrtle plant includes sugar, protein, EO, energy, fat, fiber, tannin, USFA, and saturated fatty acids as 8.64%, 4.17%, 0.01%, 11.21 kcal/g, 2.37%, 17.41%, 76.11 mg/100 g, 74.1% and 25.7% respectively (Aydin and Ozcan, 2007).

1.8 Pharmacological Effects

1.8.1 Antibacterial Effect

In an article, (Taheri, et al., 2013) documented effectivity of leaves of myrtle plant against the pathogenic microbes i.e. *Staphylococcus aureus* and *Vibrio chloreae*. In his research trial (Mansouri, et al., 2001) observed effects of extract of myrtus and EOs according to the compositions of respective chemical compounds. Myrtle plant extract and EOs were used to check effects on 6 Gram +ve microbes (*Listeria monocytogenes, Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus pneumonia, Micrococcus luteus,* and *Staphylococcus aureus*) and 4 Gram –ve microbes (*Pseudomonas aeruginosa, Escherichia coli, Campylobacter jejuni,* and *Proteus vulgaris*). The range of minimum inhibitory concentration MIC was observed from 0.1 (for *S. aureus* and *M. luteus*) to more than 2 mg/ml (for *Eschercia coli*). The antimicrobial resistance was shown by EO obtained from myrtle plant against *M. tuberculosis* in concentration of 0.17%, whereas no activity against *M. avium* subspp. *paratuberculosis* (>2%) (Zanetti, et al., 2010) and decreased activity was against *P. aeruginosa,* (Owlia, et al., 2009).

1.8.2 Antiviral Effects

The effectivity against virus is because of its direct virucidal effect which is exhibited by destroying structural proteins of virus (Djilani and Dicko, 2012). Furthermore, it is speculated that the EO either interferes with the process of viral replication resultantly interfering with viral

membranes or the EO masks viral components, which is very important for entry into host cells (Saddi, et al., 2007). There are a lot of phytobioactive chemicals compounds which act by interrupting the biosynthesis of nucleic acid (Jassim and Naji, 2003). Myrtle plant produces very important chemical compound against virus, α-caryophyllene (Djilani and Dicko, 2012).

1.8.3 Anti-inflammatory Effects

A research trial was performed on rats by using 80% ethanol extract of myrtle plant which exhibited anti-inflammatory effects by suppressing carrageenan-induced paw edema whereas the aspirin was used as standard anti-inflammatory drug (Al-Hindwai, et al., 1989). Feisst et al., (2005) reported anti-inflammatory activity due to some compounds (semimyrtucommulone, nonprenylated acylphloroglucinols and myrtucommulone). In a study on rats the ethanol extract of *Myrtus communis* L affected the biochemical and histopathological alterations which were induced by acetic acid. The extract positively reduced colitis and could also be used for treating inflammatory bowel diseases (Sen, et al., 2016). The *in-vitro* anti-inflammatory effects were tested on model of lipopolysaccharide-stimulated microbes by significantly inhibiting nitric oxide NO production without affecting cell viability (Bouzabata, et al., 2015).

1.8.4 Antidiarrheal Effects

The 70% hydroalcoholic methanol extract of myrtle plant showed antidiarrheal, bronchodilator, and vasodilator effects on isolated tissues and the effects could possibly be due to blocking of calcium channels and anticholinergic activities (Janbaz, et al., 2013). The EO extracted from myrtle leaves also contains antidiarrheal and antispasmodic activities in rats as well as in guinea pigs both *in-vivo* & *in-vitro* and such activities inhibit intestinal motility and fluid secretions (Chala, et al., 2017). In another *in-vivo* trial a remarkable dose-dependent protection against diarrhea was observed when aqueous extracts of seeds and berries of myrtle were used. The results also showed that aqueous extract protected the rat from castor-oil induced diarrhea (Jabri, et al., 2015). In another trial on mice (Sisay, et al., 2017) documented methanol 80% myrtle plant leave's extract effectively protected the animals against infectious diarrhea.

1.19 Aim of the Research Trial

Neonatal diarrhea occurs frequently in dairy calves all over the world, causing huge economic and productivity losses that undermine healthy and sustainable development of animal husbandry (El-Seedy, et al., 2016). Moreover, even if calves recover from the diarrhea, their subsequent growth and development are hindered, which later affects their productivity in adulthood (Heinrichs and Heinrichs, 2011). Generally, feed supplementation could reduce the incidence of diarrhea and improve the health of calves. Therefore, it is very important to determine the application of effective plant-based antidiarrheal agents (Wang, et al., 2018).

The objectives of this study are:

- To evaluate whether myrtle extract supplementation can reduce the incidence of diarrhea in pre-weaning calves while improving the growth performance.
- To evaluate whether the myrtle extract supplementation can improve serum immunity in pre-weaning calves.

2. MATERIAL AND METHODS

This research proposal was accepted and officially sanctioned by the Committee of Animal Care and Use, Afyon Kocatepe University, Turkey, No. 49533702/42, dated 14-04-2021. The research work was carried out at a dairy farm named as "Kaanlar Tarım ve Hayvancılık Çiftliği" located in the province of Çanakkale, Turkey.

2.1 Experimental Animals

A total of forty-eight (*n*=48) newly born calves (BW 40 ± 4 kg) of Simmental breed were selected and randomly assigned to four treatment groups with 12 calves in each group. Immediately after birth all of the newly born calves were fed colostrum 4 liters/calf/day for the initial 2 days. The calves were then weighed, brought to calf shelter or calf shed and individually housed in barley straw-bedded calf hutches (dimension of a calf hutch was 2.23 m x 1.28 m x 1.4 m). From 3rd day till the end of trial whole full fat milk 2 liters/calf was offered to all calves twice a day (08:30 am and 04:00 pm) through milk nursing bottles.

2.2 Experimental Groups

Immediately after birth the calves were ear tagged with specific tag number for identification and also for their future reference. Turn by turn after reaching the calf shelter the calves were randomly assigned to respective treatment group which included: control group (only milk), MP (milk and probiotic) group, MM (milk and myrtle) group and MMP (milk, myrtle and probiotic) group whereas each main group contained 12 calves. Every main group was further divided into 2 replicates containing 6 male and 6 female calves. The control group was given only milk, MP group was given milk and probiotic "Prolyt Pack®" 10 gm mixed in milk, the MM group was given milk and myrtle extract 50 mL, the MMP group was given milk, probiotic 10 gm and 50 mL myrtle extract. The detail of groups is given below.

Table 2.1: Groups of experimental animals and their respective supplementation

Sr. No.	Group	No. of calves		Supplementation
		Male	Female	
1	Control	6	6	Only milk 4 L/calf/day
2	MP	6	6	Milk 4 L/calf/day and probiotic "Prolyt pack®" 10 gm mixed in milk
3	MM	6	6	Milk 4 L/calf/day and myrtle plant extract @ 50 mL
4	MMP	6	6	Milk 4 L/calf/day, probiotic "Prolyt pack ®" @ 10 gm and myrtle extract @ 50 mL

2.3 Myrtle Plant Extract (Biyoderm®) Solution Preparation and probiotic

2 g stem and 2 g leaf part of the myrtle (*Myrtus communis* L.) variety grown in a certain region were first reduced in a mechanical shredder and kept in sterile distilled water with pH 4.5 at 98°C for 22 minutes. The solution prepared in this way was left to cool, and then its acidity was balanced by using Na_2HPO_4 as a buffer solution. The solution was filtered to a thickness of 0.2 mm with a medium flow ash content of < 0.01%, bottled and stored in the refrigerator. The myrtle plant extract was purchased from ARS-ARTHRO Biyoteknoloji A. Ş Ankara Turkey.

2.4 Milk feeding and additive supplementation protocol

Before milk feeding the temperature of milk was raised to 39-40 ^oC in the milk containers, the milk nursing bottles were thoroughly washed and cleaned with detergent. At every morning time the required dose of myrtle extract 50 mL/calf/day and probiotic Prolyt Pack® 10 mg/calf/day (upto 3 months of age of calf) was put in milk, the milk nursing bottles were shaken to thoroughly mix the supplements in milk and then the milk nursing bottles were served to the calves of respective treatment group. All calves were offered whole full-fat milk (from 3rd day), hay grass (from 4th week), and starter feed (from 3rd week) till the completion of trial, according to the recommendations of NRC (2001).

Table 2.2: Composition of probiotic Prolyt Pack®

Composition	Quantity
Ash	6.7%
Crude fat	0.2%
Crude protein	8.3%
Crude cellulose	0.0%
Sodium	4.1%
Zootechnical additives/kg	
Bacillus licheniformis (DSM 5749) and *Bcillus subtilis* (DSM 5750) (1:1 ratio)	6.5 x 10^{10} CFU
Enterococcus faecium (NCIMB 11181)	5 x 10^{10} CFU
Nutritional additives	
Betain (Betain anhidre) (3a920)	96000 mg
Vitamin A (3a672a)	25000 mg
Vitamin E (1 mg all-rac-alfa-tokoferil asetat = 1 IU) (3a700)	2575 mg
Niasinamid (3a315)	1000 mg
Trace elements	
Copper, glycine hydrate (3b413)	160 mg
Manganese, glycine manganese chelates (3b506)	1200 mg
Zinc, glycine zinc hydrate chelate (3b607)	1200 mg

2.5 Health Checkup of the Calves

The health status i.e. temperature, fecal consistency (to examine diarrhea), scours (feces with dry matter below 10 %), respiration (to check pneumonia), skin was examined for any ecto-parasitic infestation, anhydrosis, alopecia, any fungal infection and overall body condition of calves was daily examined to check any possible disease. During routine examination if any of the calves found diseased, it was treated accordingly by the researcher who was also a veterinarian.

In the supplemented calves no severity and less frequency with no diarrhea sequels was observed so fluid therapy was not required during whole of the duration of trial. However, in the control group severity of diarrhea was observed in calves so supportive therapy was given by injecting

drip of DekstroVIP 5% 1000 mL through intravenous i/v route. The supportive therapy was repeated if the calf was severely dehydrated.

In trial whichever the calf, irrespective of the supplemented or non-supplemented group, affected by diarrhea were treated per-orally with Kaopektin 80-120 mL/calf, parenterally with antibiotics Marbiotic 10% 2 mL/25 kg body weight and Meloxicam 2.5 mL/100 kg body weight through intramuscular i/m route. The treatment was continued for 3 consecutive days.

2.6 Cleaning of the Calf Hutches

After every 3-4 day the wet bedding was changed and the calf hutch was thoroughly cleaned. At least for one time in entire trial period every calf hutch and the area in front of the hutch was thoroughly washed with the help of water and air pressure gun. In the wall of calf pen there was a hole, for insertion of milk bottle, it was routinely cleaned and disinfected.

2.7 Research Parameters

2.7.1 Calf Starter Feed

During the trial when the calves were brought to calf shed the feed was not offered to them in the 1^{st} week and in the beginning of 2^{nd} week the un-weighed feed was offered to the calves just to make them familiar with the feed (adaptation period). In the beginning of 3^{rd} week (from the 15^{th} day), weighed and fresh feed was offered to the calves every day. On next day the remaining feed was collected from the feed pots and stored in air sealed empty bags specific for every calf and this practice kept on going for next 7 days. On the 8^{th} day the bags, having remaining feed of previous 7 days, were weighed. The ingredients and chemical composition of the calf starter concentrates are shown in Table 6.

On every morning fresh and clean water was offered to all calves starting from day 1, grass hay was offered to calves in the beginning of 4^{th} week, they started to nibble and later on gradually started to eat grass hay, both water and hay remained available *ad-libitum* throughout the duration of trial.

2.7.2 Body Weight Measurement

The body weight measurement of all calves was performed for 3 times; initial weight (at the time of birth), weight at 1 month and final weight at 75th day of trial (at the time of weaning). On the day of weight measurement before the morning feeding the calf was brought to the weighing balance and its weight was measured with the help of an electric weighing scale.

2.7.3 Blood Sampling

On the 7th, 20th, 40th, 60th and 75th day of trial, 10 ml blood from every calf was drawn from the jugular vein and immediately preserved into specific vacutainers. There were two types of blood samples taken from every calf by the researcher; one blood sample (uncoagulated) was taken in vacutainers having anticoagulant (ethylenediamine tetra acetic acid EDTA). The analysis of whole blood included (hematocrit HCT, mean corpuscular volume MCV, platelets PLT, hemoglobin Hb, mean corpuscular hemoglobin concentration MCHC, white blood cell WBC, red blood cell RBC, mean corpuscular hemoglobin MCH, red cell distribution width RDW, Neutrophil, Lymphocyte, Monocyte, Eosinophile, Basophile) was performed immediately after blood collection using the kits related to the Mindray BC 2800Vet device.

The other blood sample (coagulated) was taken in a vacutainer having no EDTA, later on it was centrifuged at 5000 rpm (revolutions per minute) for 10 minutes with the help of a centrifuge machine to obtain serum samples. The serum samples were frozen at -20°C, thawing of serum samples was done at room temperature and later on analyzed for total protein, beta hydroxybutyric acid BHBA, blood urea nitrogen BUN, glucose, non-esterified fatty acid NEFA, and immunoglobulin G with the help of relevant kits in the Chemwell (2910) automatic analyzer.

2.7.4 Fecal Consistency Score

During the trial every day in the morning time before feeding the feces of all calves were checked by the researcher and rated accordingly to assess the consistency, where 0 was rated as normal, 1 was rated as semi formed or pasty, 2 was rated as loose but having enough consistency to stay on bedding material and 3 was rated as watery in texture which leaked through the bedding material

and the fecal score was rated according to (Heinrichs, et al., 2003). The calves showing symptoms of diarrhea were immediately treated by the researcher. The recovery from diarrhea was assessed by observing the fecal consistency score, if the fecal consistency score was ≤1 for two consective day then the calf was declared as healthy having no diarrhea.

The %age of diarrhea rate was calculated with the help of following formula (Longstreth, et al., 2006).

$$\text{Diarrhea rate \%} = \frac{\text{total calves with diarrhea per day}}{\text{total no.of calves x no.of days examined}} \text{X } 100$$

The feed conversion rate FCR was calculated by using following formula

$$\text{FCR} = \frac{\text{feed consumed (g)}}{\text{weight gain (g)}}$$

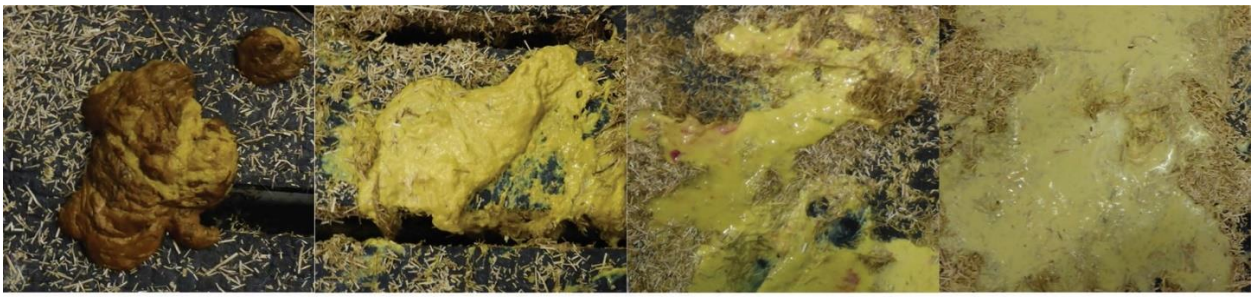


Figure 2.1: Fecal consistency score

2.8 Vaccination

All calves were vaccinated with 5 ml Bovilis (Bovipast, MSD) subcutaneously from the neck region to protect them from respiratory system diseases when they reached the age of 21 days. The booster of the vaccine was made 21 days after the first administration (when the calves were 42 days old) in the same way and dose. The vaccine contains inactive BRS virus (EV 908) with a TCID50 value of 105.5, inactive Parainfluenza 3 virus with a TCID50 value of 105.5 (SF-4 Reisinger strain) and 9X109 inactive *Pasteurella haemolytica* cells (serotype A1).

Table 2.3: Vaccination schedule

Vaccine name	Dose rate and route	Age	Protection against
Coglavax	2 ml, s/c	5th & 26th day	*Clostridium perfringen* type A, B, C, and D. *Clostridium septicum* *Clostridium tetani* (tetanus)

			clostridial infections, pulpy kidney disease, gas gangrene, nutritional dysentery,
Bovipast, MSD	5 ml s/c	21st & 42nd day	Respiratory diseases
Hiprovis 4 and VBR 3	2 ml s/c	41st & 56th day	Bovine viral diarrhea BVD, parainfluenza-3, infectious bovine rhinotrecheitis IBR, *Pasteurella multocida*.
Şap (Foot and Mouth Disease FMD)	2 ml s/c	71st day	FMD virus type A, O and Asia-1.

2.9 Analyses

2.9.1 Milk Analysis

The total duration of the trial was 224 days (17-02-2022 to 28-09-2022) and every day in the morning the milk was taken from milk tank and analyzed and there were 240 replicates. Tank milk analyzes were made in the milkana test device.

- Protein; 3.19 %
- Lactose; 4.83 %
- Fat; 3.35 %

2.9.2 Feed Analysis

The proximate analysis was performed for the feed samples which included calf starter feed and grass hay were done according to AOAC (1990).

Table 2.4: Composition of grass hay

Composition	**Quantity**
Dry matter	89.9%
Crude protein	15.7%
Crude fat	2.1%

Ash	9.2%
NDF	50.9%
ADF	35.4%

2.10 Production, Validation Processes and Chemical Components of the Extract

Active ingredients (mg/L) of the *Myrtus communis* L plant extract (MPE) used in the study is presented in Table 4. Myrtus plant extract (Bioderm®, ArsArthro Biotechnologies Inc, Ankara, Turkey) was carried out in a process including assortment, grinding, drying, and verification of plant material, purification, removal and drying (Handa, et al., 2008).

Concisely, during this water-based extraction no solvent was used except distilled water (99% pure water as a carrier and 1% MPE) and it was performed on dehydrated leaves and barks of plants. The extraction was water-based and no solvent was used except distilled water (1% MPE, 99% pure water as a carrier). Liquid chromatograph (LC-MS/QTOF-Agilent Technologies, Santa Clara, CA, USA) and Mass Spectrometer analysis instrument (Orbitrap-Thermo Electron, Bremen, Germany) were used to determine active ingredient composition. Moreover, Agilent Poroshell 120 ECC183.0x100 mm, 2.7 µm colon were also used by 10Mm ammonium formate 0.1%, formic acid and water with MFB 0.1% formic acid MeOH (Gas Temperature 325°C, Gas Flow 10 L/min, Nebuliser 45 psig) for active ingredients determination (Agilent Technologies, Santa Clara, CA, USA). All the ingredients were reported by negative and positive polarization with both instruments. Amino acid analyses by using HPLC device according to Bruckner and Westhauser, (2003). Mineral analyses were carried out on Shimadzu ICPMS-2030 system equipped with a mini torch for low Ar gas consumption.

2.11 Statistical Analysis

The BW, FI, FCR, biochemical and hematological results were evaluated using JMP statistical pocket program (JMP, 2003) as GLM. All data was expressed least square means in the tables. Significance level was set at ($p \leq 0.05$).

3. RESULTS

3.1 Chemical Composition of *Myrtus communis* L plant extract

All the contents of the myrtle plant obtained as a result of the analysis are shown in below mentioned table.

Table 3.1. Active ingredients of *Myrtus communis* L plant extract

Amino Acid	**(mg/L)**	**Mineral**	**(mg/L)**
Arginine	634,54	Cadmium (Cd)	0.002
Alanine	691,27	Lead (Pb)	0.007
Cystine	218,82	Arsenic (As)	0.003
Aspartic Acid	668,01	Mercury (Hg)	0.03
Glycine	417,49	Magnesium (Mg)	27000
Glutamic Acid	1767,92	Sodium (Na)	324000
Isoleucine	414,90	Iron (Fe)	7.1
Histidine	1133,84	Tin (Sn)	1
Lysine	0,00	Aluminium (Al)	1.35
Leucine	173,50		
Proline	16254,60	**Chemical Composition**	**(mg/L)**
Methionine	281,53	Myricetin	15.34
Threonine	0,00	Quercetin	0.19
Serine	182,91	Catechin	4.80
Valine	151,64	Salicylic acid	0.06
Trytophan	907,13	Gallic acid	0.13
Phenylalanine	895,06	Rosmarinic acid	0.01
Tyrosine	1003,04		
Norvaline	72,46		

3.2 Chemical Composition of Calf Starter Feed

The ingredients of calf starter feed are mentioned below.

Table 3.2. Chemical composition and ingredients of calf starter

Feedstuff	***% DM***
Finely ground corn grain	26
DDGS (Corn)	15
Soybean meal (48% CP)	14.7
Corn Bran (18% CP)	12.6
Rasmol	12
Wheat grain, coarse ground	8
Sunflower meal (Dehulled, 36% CP)	7.9
Limestone	2.9
Salt	0.7
Premix*	0.2
Chemical Composition	
Dry Matter, %	88.19
Crude Protein, %	20.03
Metabolizable energy (KCal/kg)	2.622
Crude Fat, %	3.65
Crude Cellulose, %	7.39
Crude Ash, %	7.62
Ca, %	1.28
P, %	0.53
NDF, %	20.21
ADF, %	8.64

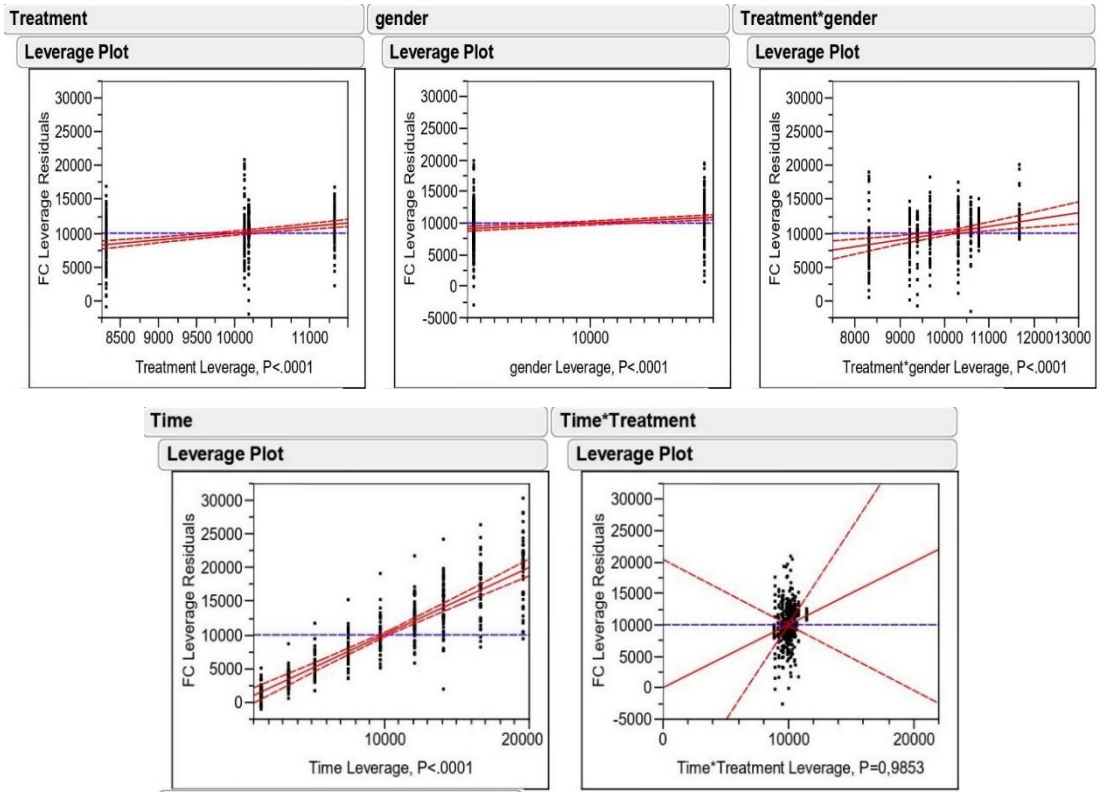
* 1 kg of Premix includes; 15000000 IU of Vitamin A, 3000000 IU of Vitamin D_3, 40000 mg of Vitamin E, 100000 mg of manganese, 100000 mg of iron, 100000 mg of zinc, 20000 mg of copper, 1600 mg of iodine, 300 mg of cobalt, 300 mg of selenium

3.3 Performance parameters

3.3.1 Starter consumption

The statistical results showing effects of supplementation on feed consumption are shown in graph 1. The treatment calves had a higher feed consumption (p=0.0001) values and between treatment groups the MM had statistically significant feed consumption. During the 1st month a relatively lower feed consumption was observed whereas the increased feed consumption was noted in the 2nd month. Overall it was found that supplementation and male gender had positive effects on the feed consumption. Furthermore, it was also noted that the feed consumption was increased as the time progressed.

Graph 3.1: Effects of myrtle plant extract supplementation on feed consumption with respect to treatment, gender, treatment*gender, time and treatment*time

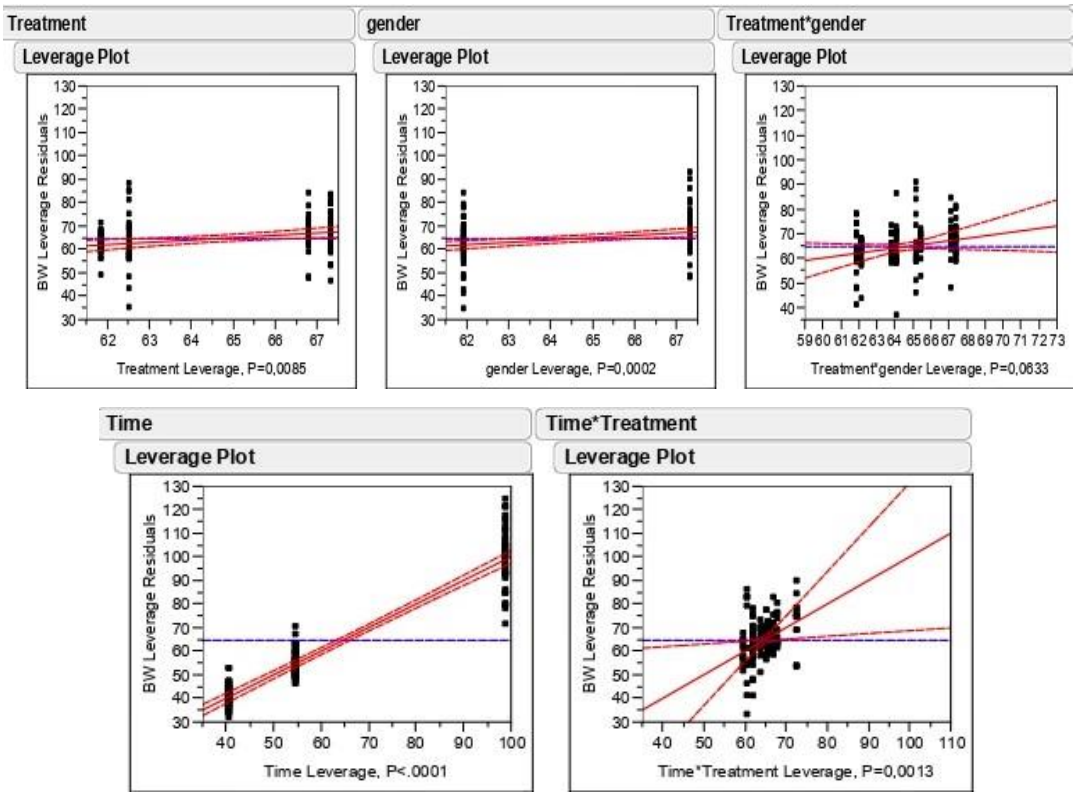


3.3.2 Body Weight Gain

The statistical results showing effects of supplementation on body weight gain are presented in graph 2. During trial it was observed that all the calves of all groups had a gradual enhancement in weight gain but *(p=0.0085*) statistically significant improvement was observed in live weights of treatment calves. On the other hand, calves belonging to MM and MMP groups had better live weight gain as compared to other two treatment groups. The ratio of weight gain was more in 2nd month as compared to that in the 1st month of the age of calves. Similarly, a significant increase in

live weight was observed in male calves as compared to the female calves of all groups (treatment*gender interaction). The supplementation had significant effects on time*treatment interaction which means that the calves grew bigger as the time passed.

Graph 3.2: Effects of myrtle plant extract supplementation on body weight gain with respect to treatment, gender, treatment*gender, time and treatment*time.



3.3.3 Feed Conversion Rate

The statistical results showing effects of supplementation on feed conversion ratio are presented in graph 3. During the experiment, the addition of myrtle extract and probiotics had no significant effect on FCR values. On the other hand, significant differences were found in the interaction of time effect (p=0.0001) and treatment*time (p=0.0036). In addition, it was observed that gender did not significantly affect the feed conversion ratio.

Graph 3.3: Effects of myrtle plant extract supplementation on feed conversion ratio relative to treatment, gender, treatment*gender, time and treatment*time.

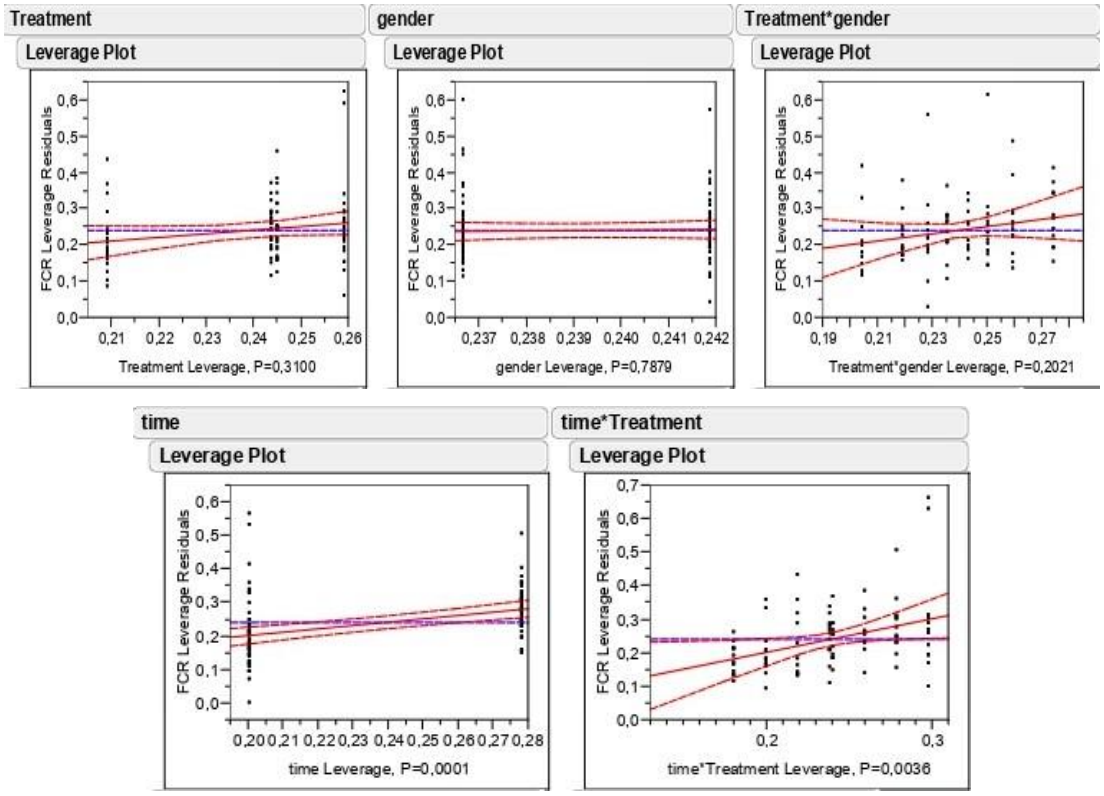


Table 3.3. Effect of myrtle plant extract and probiotic supplementation on performance of calves from birth to 75 days of age[1]

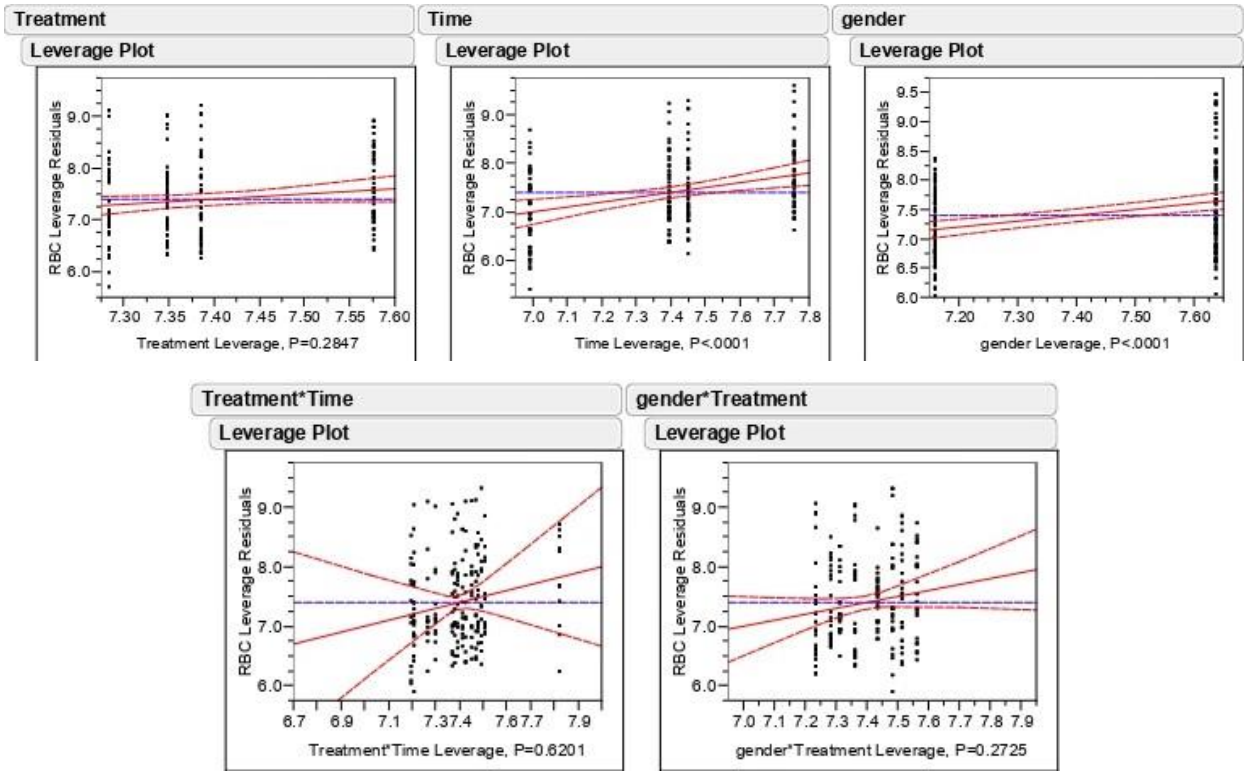
	Treatment[4]					P-values				
Item	**Control**	**MP**	**MM**	**MMP**	**SEM[5]**	**Treatment**	**Time**	**Treatment× Time**	**Gender**	**Treatment× Gender**
BW[2], kg	61.84[b]	62.51[b]	66.79[a]	67.31[a]	1.40	0.0085	<.0001	0.0034	0.0002	0.0633
Feed Intake[3], g	10195.2[b]	8309.2[c]	11329.1[a]	10137.0[b]	307.91	0.0001	<.0001	0.9853	<.0001	<.0001
FCR[4],g feed/g BW[2]	0.24	0.20	0.25	0.24	0.019	0.3100	0.0001	0.0036	0.7879	0.2021

[1] Data are represented as least square means, Control: milk without supplement, (MP):10 mg/day/head probiotic supplemented milk, (MM):50 ml/day/head Myrtus extract supplemented milk, MMP 10 mg/day/head probiotic + 50 ml/day/head Myrtus extract leaf supplemented milk.
[2] Body weight
[3] Feed intake
[4] Feed conversion ratio
[5] Standard error of mean
[a,b,c] Values with different superscripts in the same row are significantly different ($P \leq 0.05$) and for tendency declared at $P < 0.15$

3.4 Blood Parameters

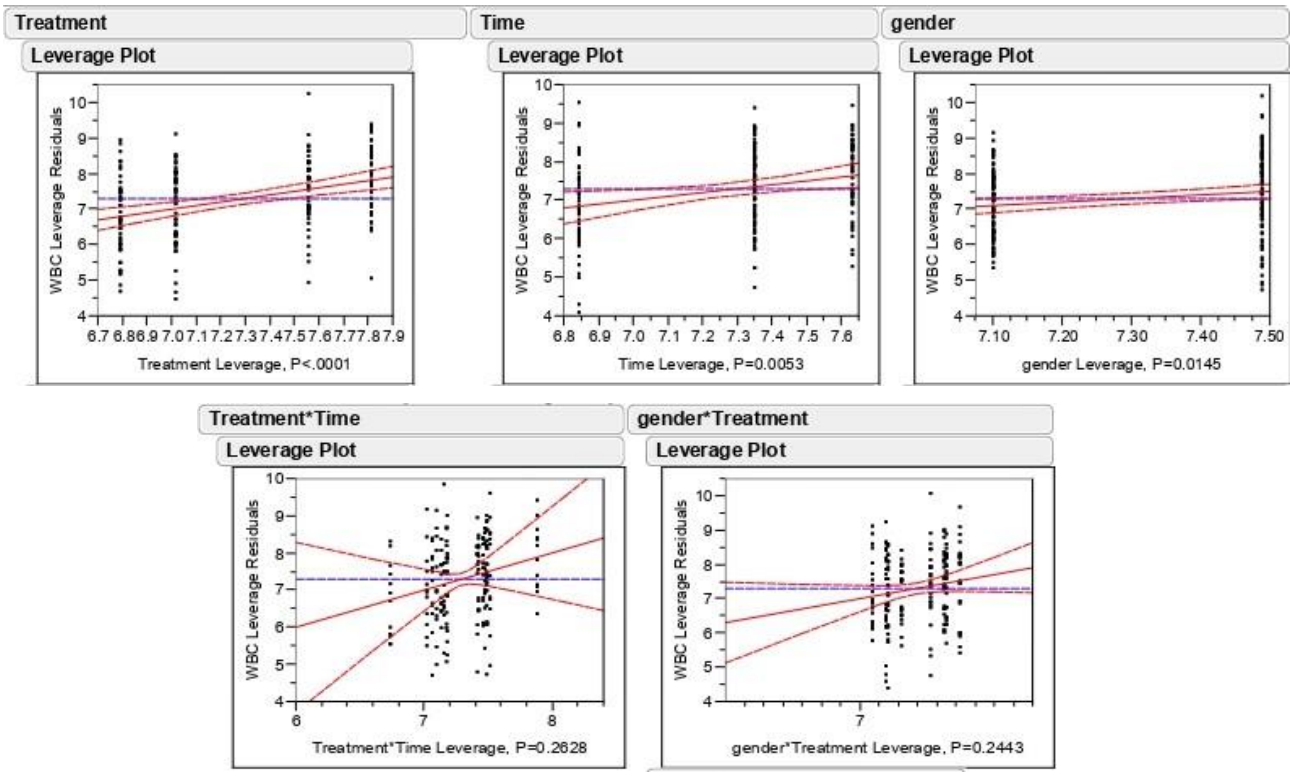
3.4.1 Blood Physiological Parameters

Graph 3.4: Effects of myrtle plant extract supplementation on red blood cells



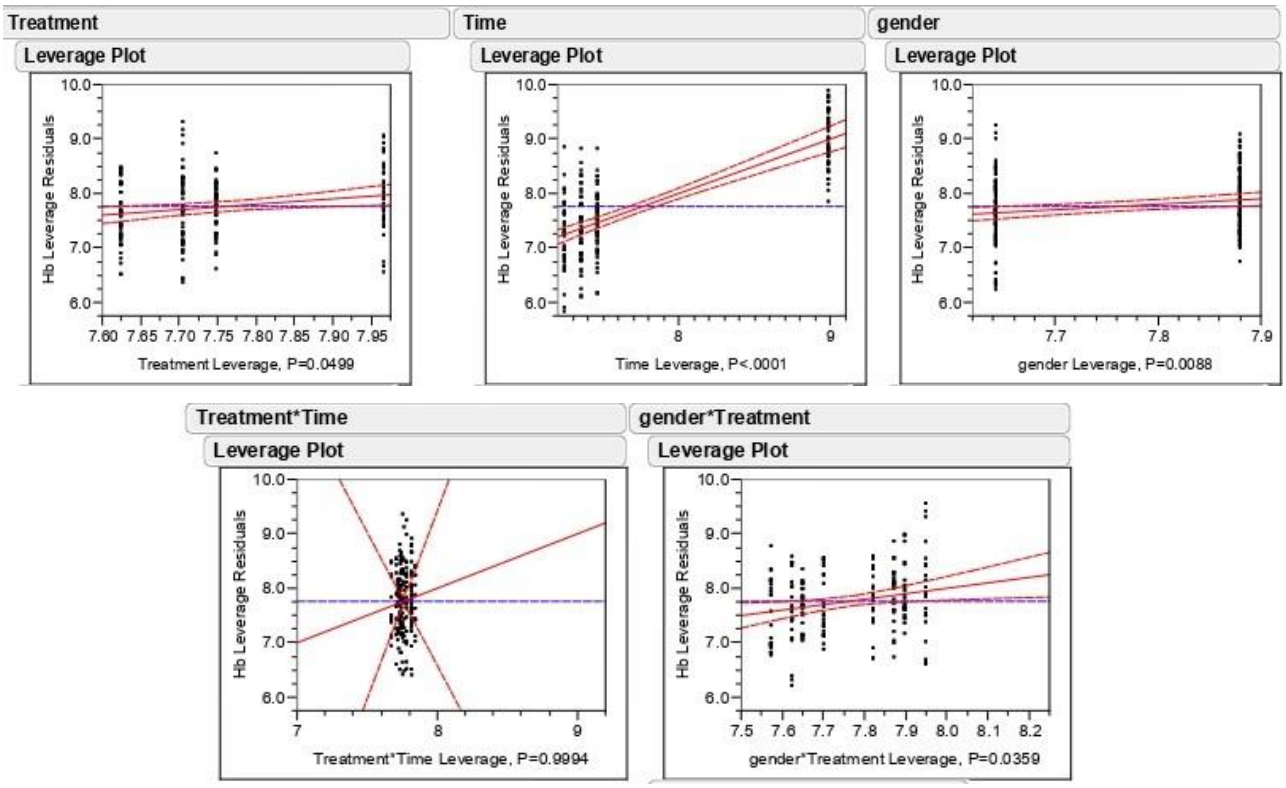
The statistical results showing the effects of supplementation on red blood cell concentration are presented in graph 4. The supplementation or treatment had 2 type of interactions (gender and time) on which the effect was assessed. The supplementation did not affect the red blood cell concentration in treatment*time interaction (P=0.6201) and treatment*gender interaction (0.2725).

Graph 3.5: Effects of myrtle plant extract supplementation on white blood cells



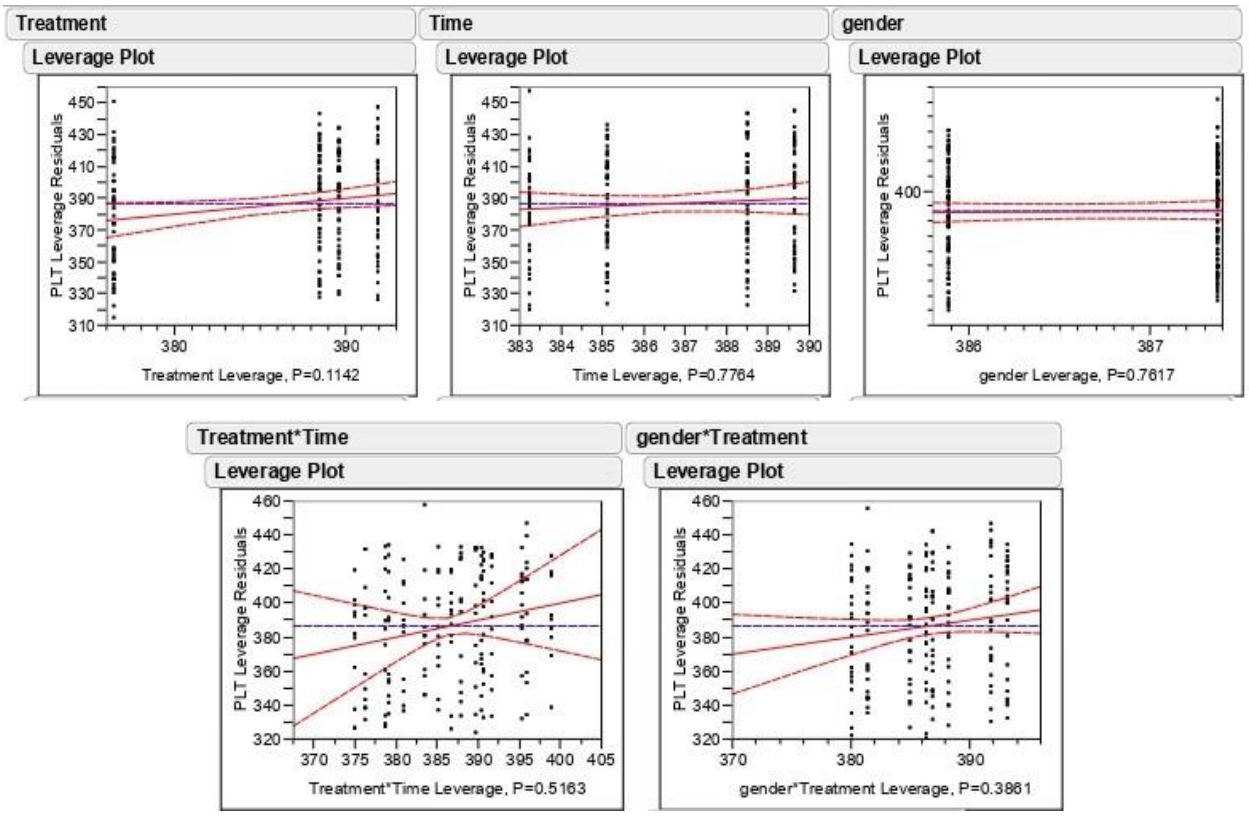
The statistical results showing the effects of supplementation on white blood cell concentration are presented in graph 5. The results showed that the supplementation did not affect the white blood cell concentration with respect to treatment*time interaction (0.2828) and treatment*gender interaction (0.2443).

Graph 3.6: Effects of myrtle plant extract supplementation on hemoglobin



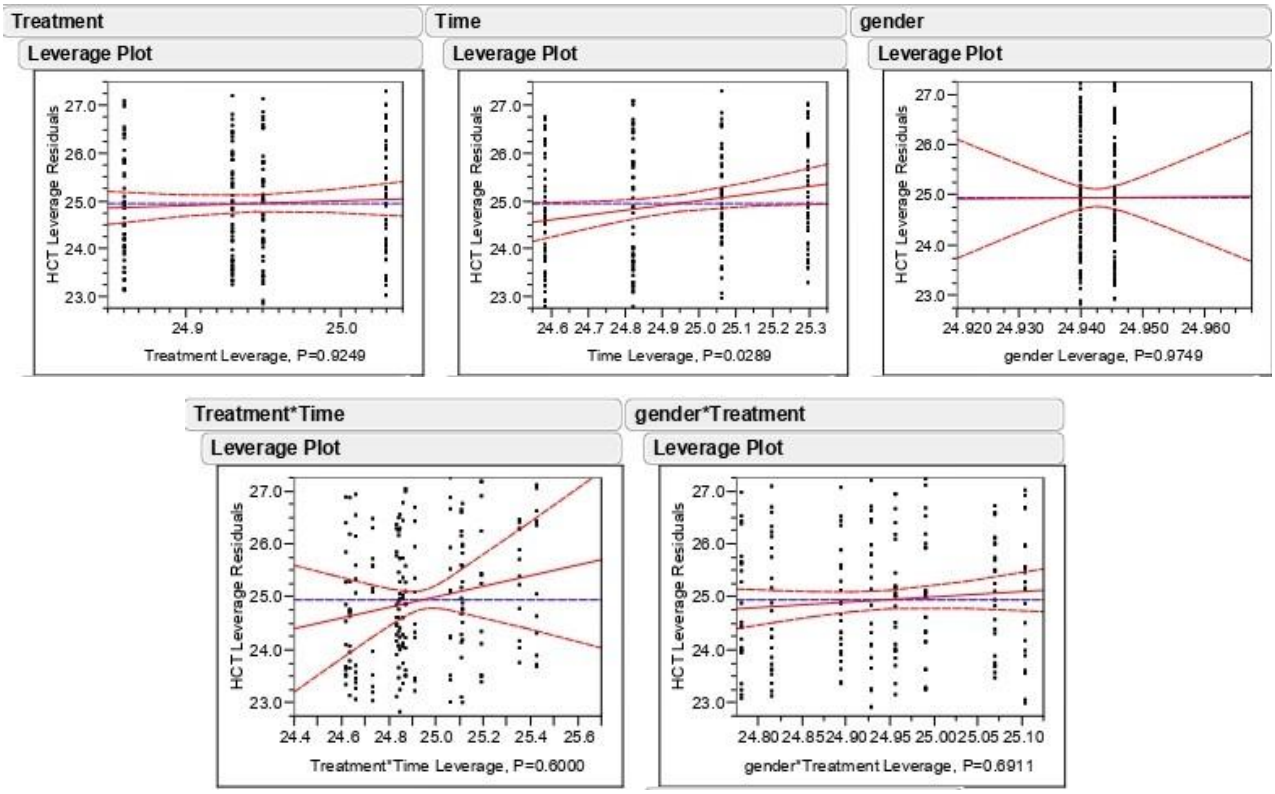
The statistical results showing the effects of supplementation on hemoglobin are presented in graph 6. The results depicted that the supplementation did not affect the hemoglobin with respect to treatment*time interaction (P=0.999) whereas the supplementation significantly affected the white blood cell with respect to treatment*gender interaction (P=0.0359).

Graph 3.7: Effects of myrtle plant extract supplementation on platelets



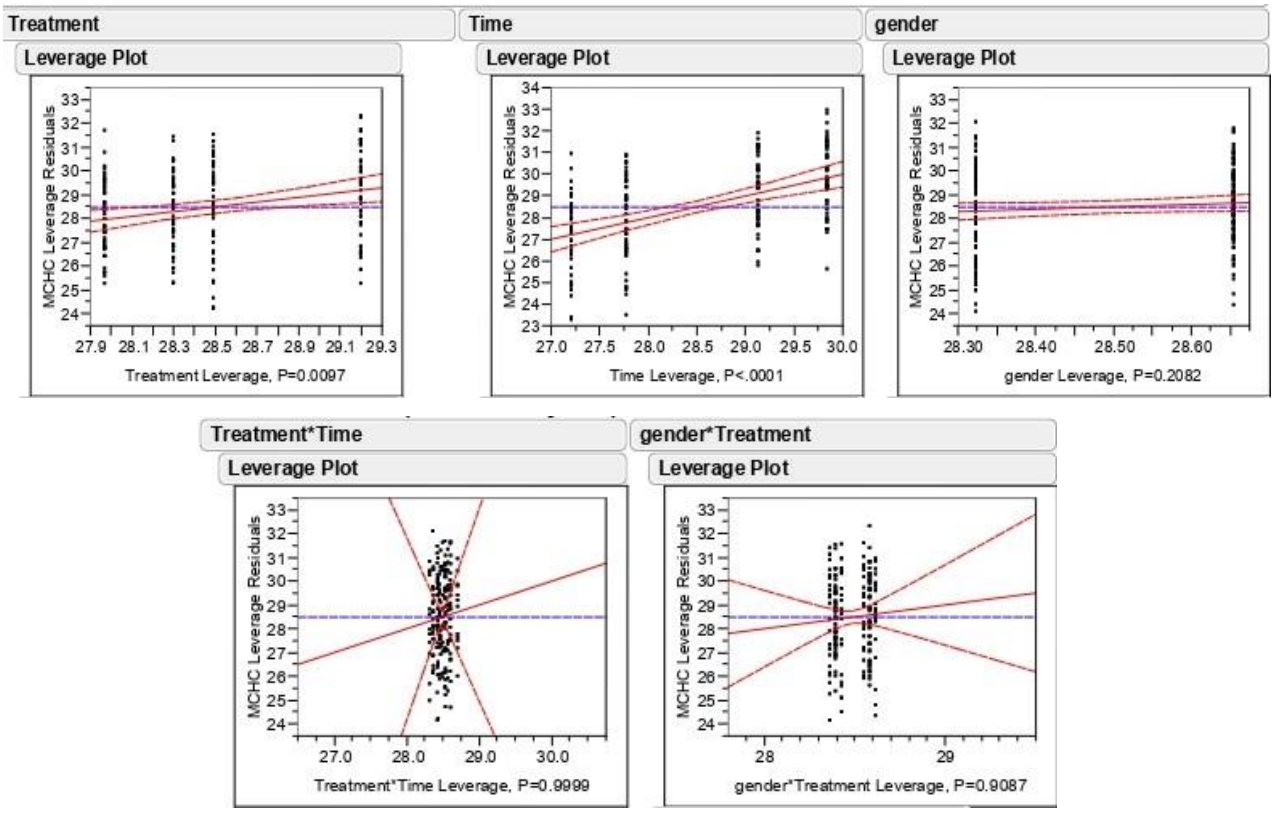
The statistical results showing the effects of supplementation on platelets are presented in graph 7. Regarding the platelet concentration, the treatment*time interaction (P=0.5160) as well as treatment*gender interaction (P=0.3861) both were not affected by the supplementation.

Graph 3.8: Effects of myrtle plant extract supplementation on hematocrit value



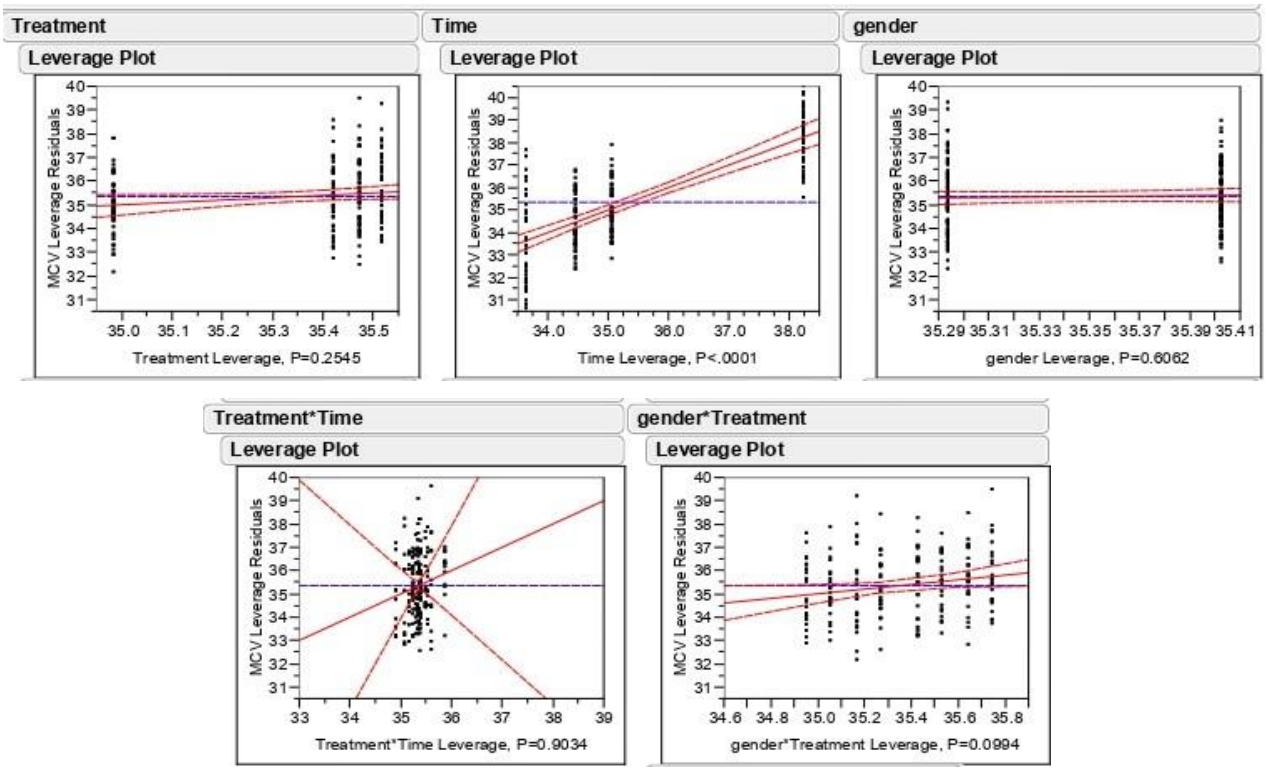
The statistical results showing the effects of supplementation on hematocrit value are presented in graph 8. The probiotic and phytobiotic supplementation did not affect both of the interactions, treatment*time interaction (P=0.6000) and treatment*gender interaction (P=0.6911).

Graph 3.9: Effects of myrtle plant extract supplementation on mean corpuscular hemoglobin concentration



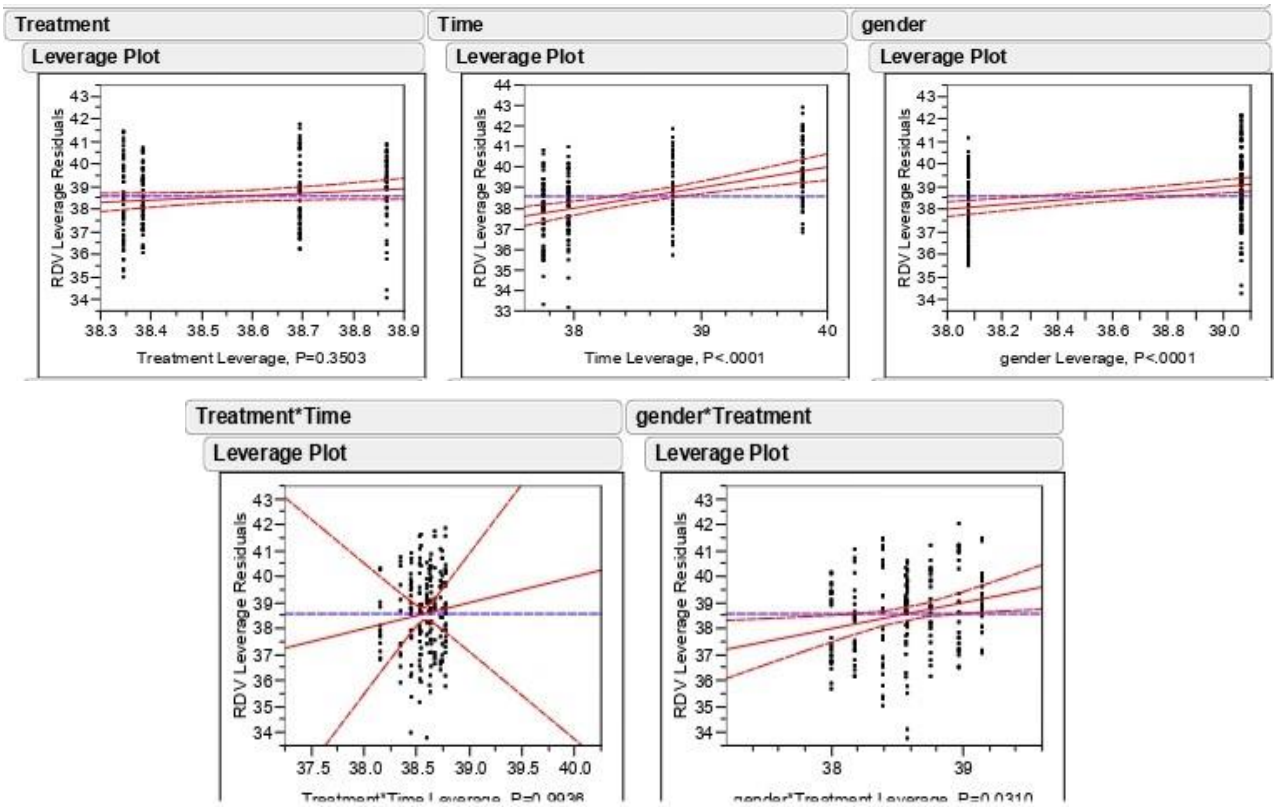
The statistical results showing the effects of supplementation on mean corpuscular hemoglobin concentration are presented in graph 9. In the results we observed that both of the interactions were not affected by the myrtle extract an d probiotic supplementation, treatment*time interaction was (P=0.9999) and treatment*gender interaction was (P=0.9087).

Graph 3.10: Effects of myrtle plant extract supplementation on mean corpuscular volume



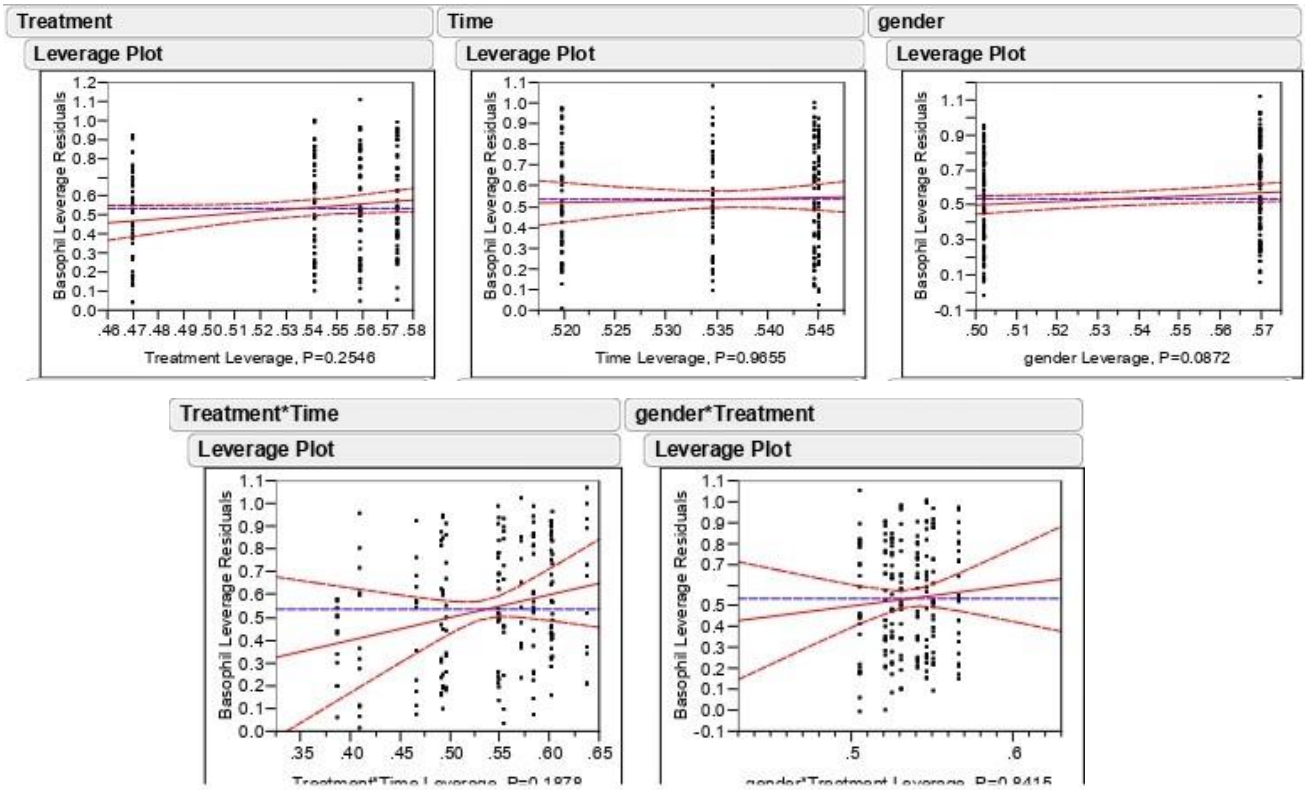
The statistical results showing the effects of supplementation on MCV are presented in graph 10. The treatment*time interaction was found (P=0.9034) and treatment*gender interaction was found (P=0.0994), both of the interactions were not affected by the supplementation.

Graph 3.11: Effects of myrtle plant extract supplementation on red cell distribution width



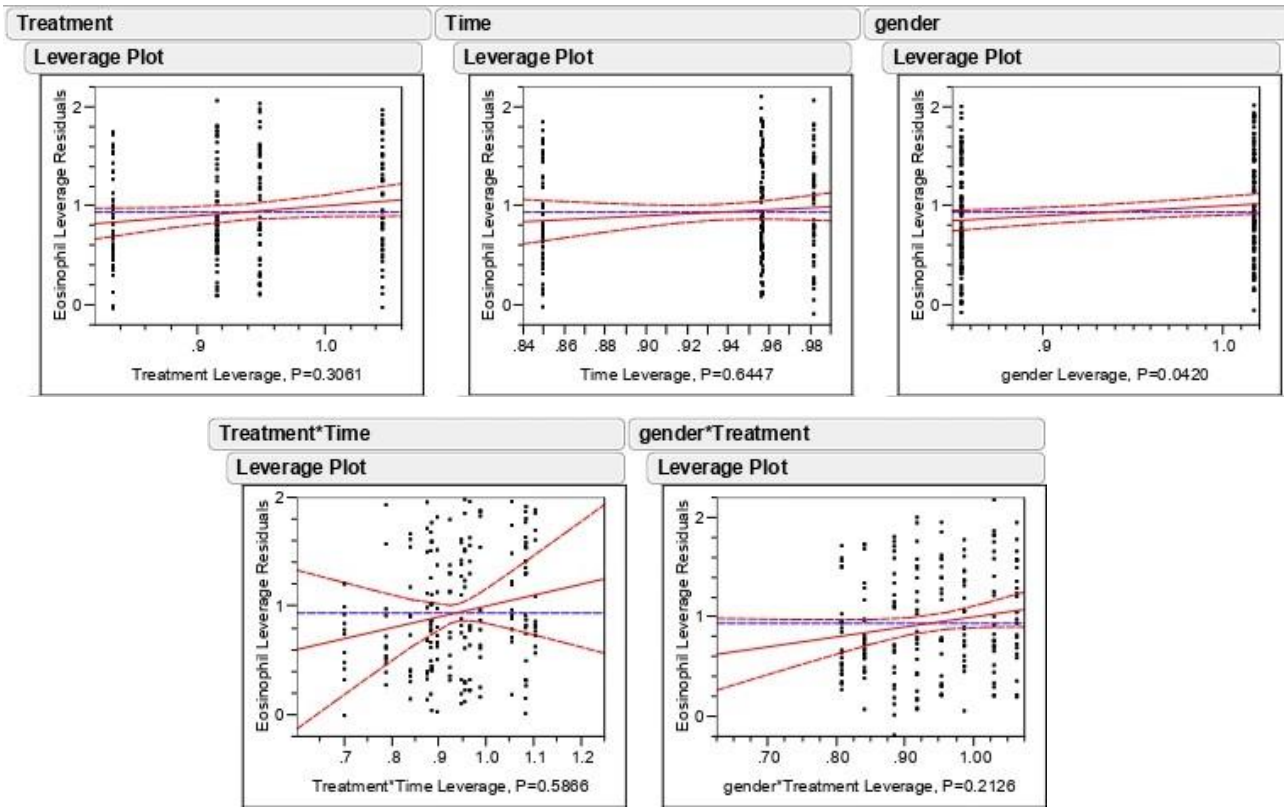
The statistical results showing the effects of supplementation on RDW are presented in graph 11. The treatment*time interaction was found (P=0.9038) and treatment*gender interaction was found (P=0.0310), both of the interactions were not affected by the supplementation.

Graph 3.12: Effect of myrtle plant extract supplementation on basophils



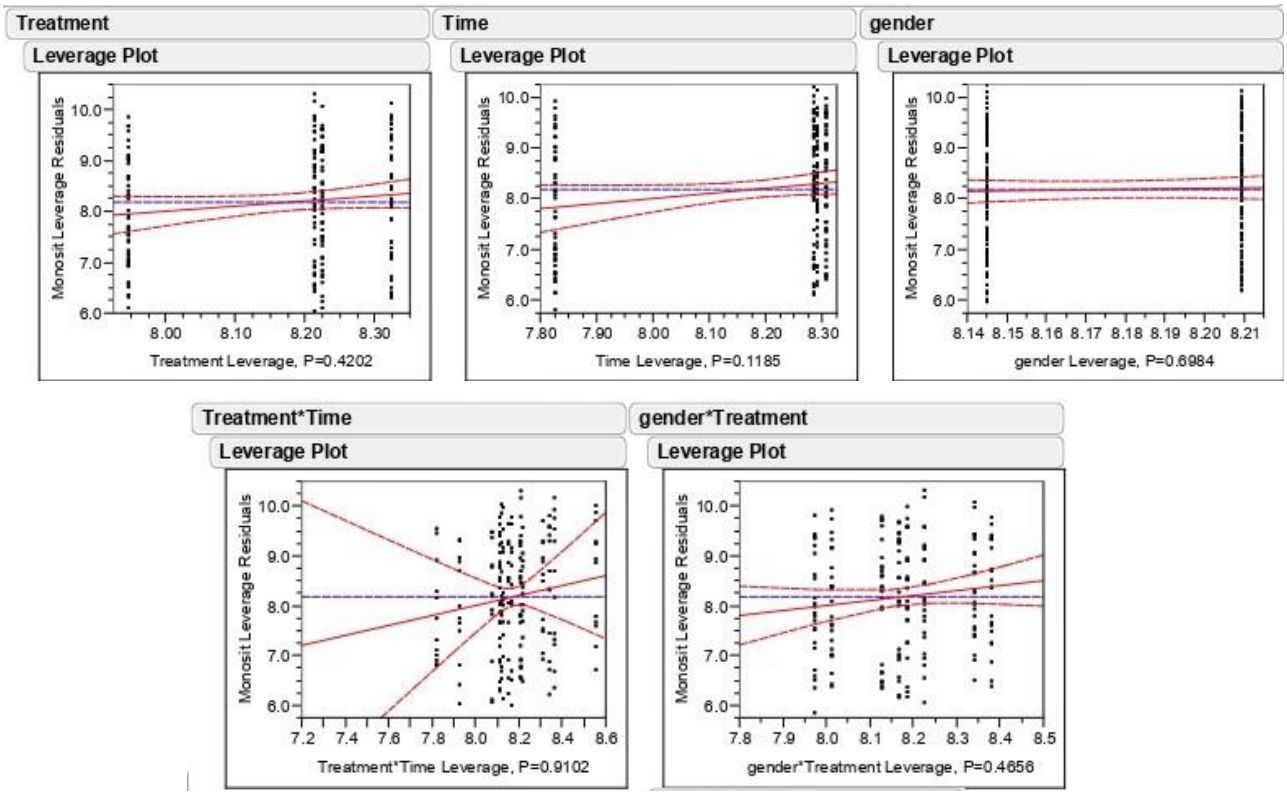
The statistical results showing the effects of supplementation on basophil are presented in graph 12. The treatment*time interaction was found (P=0.1978) and treatment*gender interaction was found (P=0.9415), both of the interactions were not affected by the supplementation.

Graph 3.13: Effects of myrtle plant extract supplementation on eosinophils



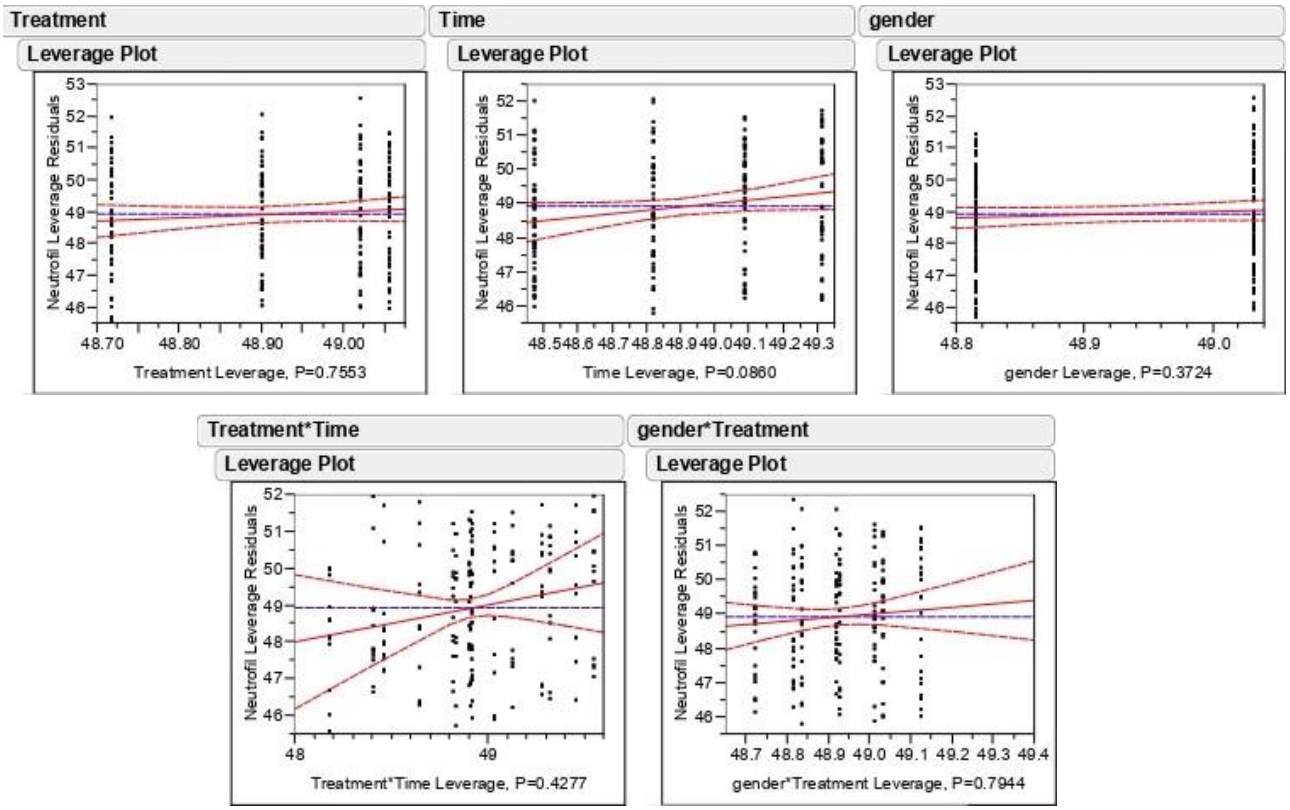
The statistical results showing the effects of supplementation on eosinophil are presented in graph 13. The treatment*time interaction was found (P=0.5866) and treatment*gender interaction was found (P=0.2126), both of the interactions of eosinophil concentration were not affected by the supplementation.

Graph 3.14: Effects of myrtle plant extract supplementation on monocytes



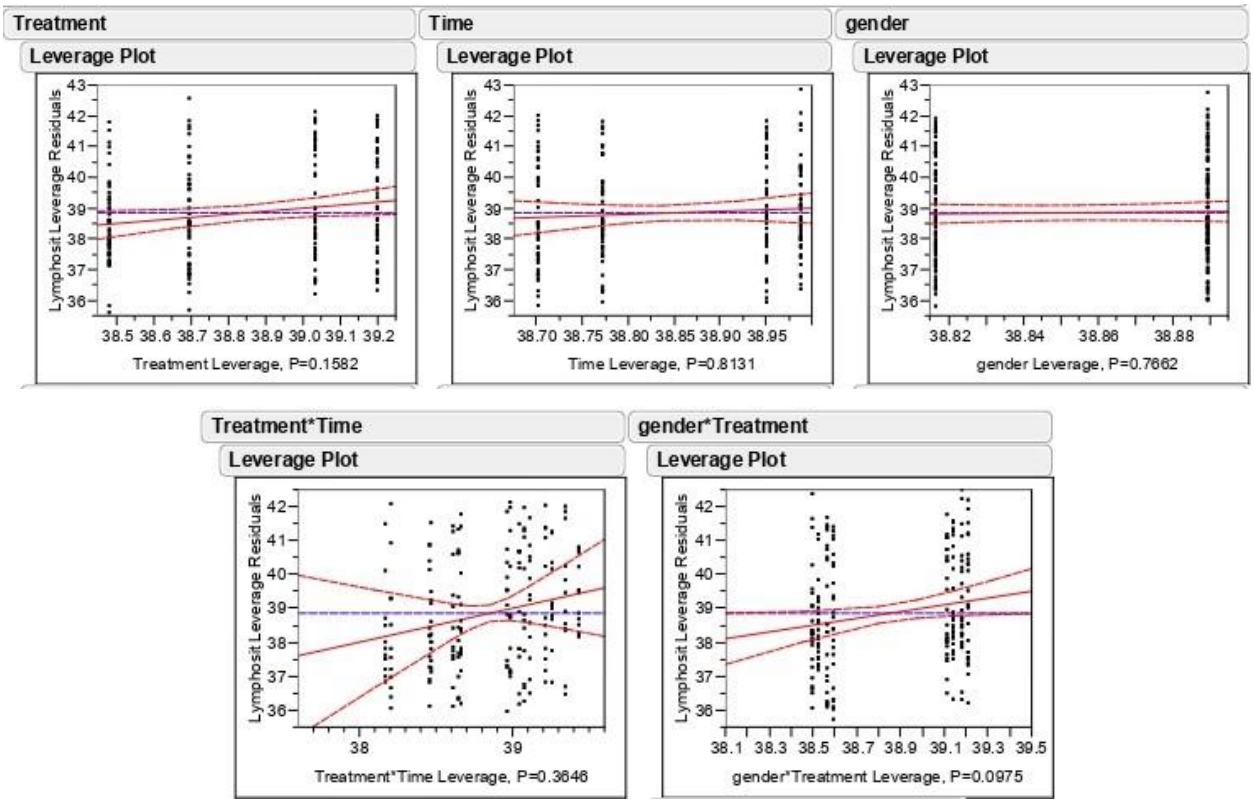
The statistical results showing the effects of supplementation on monocytes are presented in graph 14. The treatment*time interaction was found to be (P=0.9102) and treatment*gender interaction was found to be (P=0.4656), both of the interactions of monocyte concentration were not affected by the supplementation.

Graph 3.15: Effects of myrtle plant extract supplementation on neutrophils



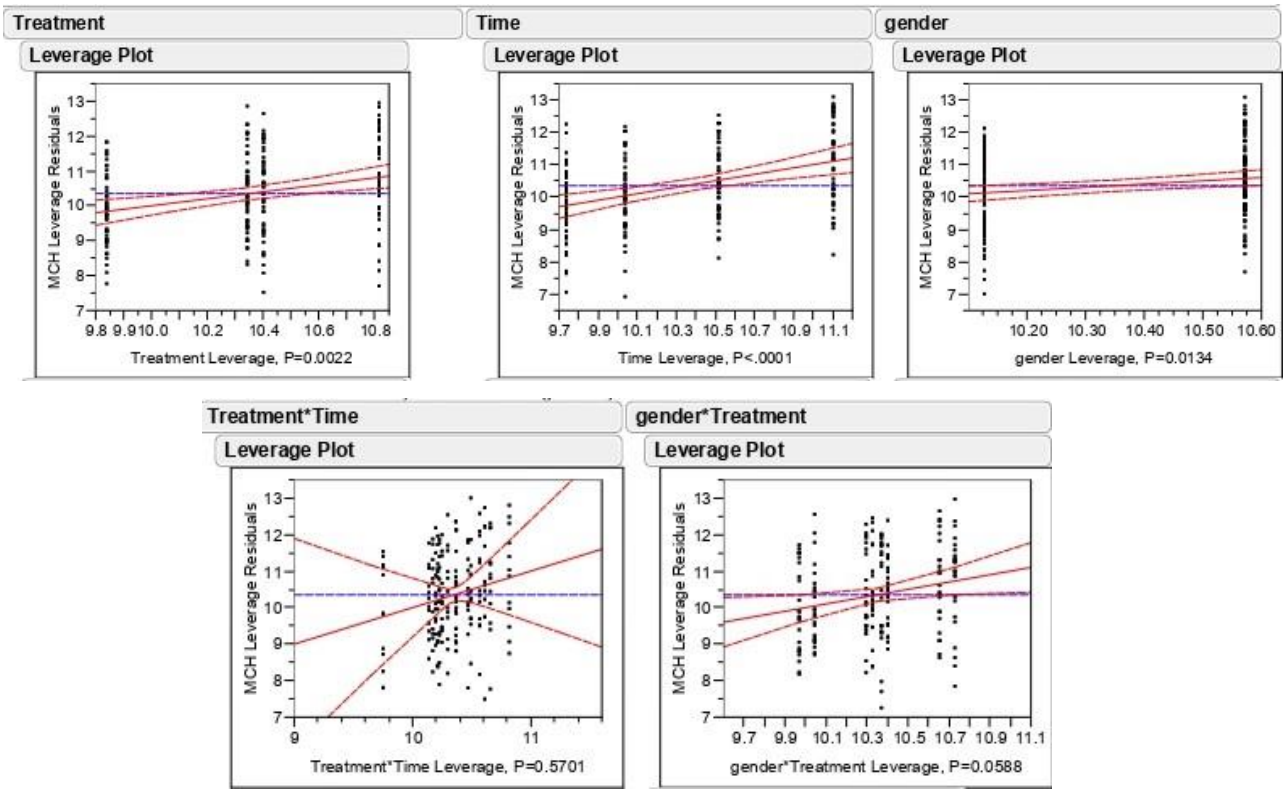
The statistical results showing the effects of supplementation on neutrophils are presented in graph 15. The treatment*time interaction was found (P=0.4277) and treatment*gender interaction was found (P=0.7944), both of the interactions of neutrophil concentration were not affected by the supplementation.

Graph 3.16: Effects of myrtle plant extract supplementation on lymphocyte



The statistical results showing the effects of supplementation on lymphocytes are presented in graph 16. In the lymphocyte concentration the treatment*time interaction was found to be (P=0.3646) and treatment*gender interaction was found to be (P=0.0975), both of the interactions were not affected by the supplementation.

Graph 3.17: Effects of myrtle plant extract supplementation on mean corpuscular hemoglobin



The statistical results showing the effects of supplementation on mean corpuscular hemoglobin are presented in graph 17. In the MCH parameter, the treatment*time interaction was found (P=0.5701) and treatment*gender interaction was found (P=0.0588), both of the interactions of MCH concentration were not affected by the supplementation.

Table 3.4: Effect of myrtle plant extract and probiotic supplementation on whole blood counts of calves from birth to 75 days of age[1]

Item	Treatment[4]					P-values				
	Control	MP	MM	MMP	SEM[8]	Treatment	Time	Treatment ×Time	Gender	Treatment× Gender
RBC[2], 10^6/Dl	7.3479[a]	7.2833[a]	7.5764[b]	7.3852[c]	0.111	0.2847	<.0001	0.6201	<.0001	0.2725
Hematocrit, %	24.9505	25.0293	24.9304	24.8606	0.175	0.9249	0.0289	0.6000	0.9749	0.6911
Hemoglobin, g/Dl	7.9662	7.7050	7.6243	7.7483	0.089	0.499	<.0001	0.9994	0.0088	0.0359
MCV[3], Fl	35.4202	34.9831	35.4722	35.5166	0.210	0.2545	<.0001	0.9034	0.6062	0.0994
MCH[4], pg	10.4008[a]	9.8393[b]	10.3427[a]	10.8147[a]	0.177	0.0022	<.0001	0.5701	0.0134	0.0588
MCHC[5], g/Dl	28.4904[ab]	27.9677[b]	29.1975[a]	28.2981[b]	0.262	0.0097	<.0001	0.9999	0.2082	0.9087
RDW[6], %	38.6937	38.3452	38.3833	38.8656	0.238	0.3503	<.0001	<.0001	0.9936	0.0310
Platelet, 10^9/L	389.643	391.922	388.511	376.439	4.893	0.1142	0.7764	0.5163	0.7617	0.3861
WBC[7], 10^9/L	7.0168[b]	6.7914[b]	7.8129[a]	7.5579[a]	0.156	<.0001	0.0053	0.2628	0.0145	0.2443
Neutrophil, %	48.7177	49.0206	49.0560	48.9008	0.242	0.7553	0.0860	0.4277	0.3724	0.7944
Lymphocytes, %	38.4814	39.0341	39.2004	38.6962	0.245	0.1582	0.8131	0.3646	0.7662	0.0975
Monocytes, %	8.2247	8.2135	7.9466	8.3239	0.166	0.4202	0.1185	0.9102	0.6984	0.4656
Eosinophil, %	1.0450	0.9158	0.8345	0.9491	0.079	0.3061	0.6447	0.5866	0.0420	0.2126

[1] Data are represented as least square means, Control: milk without supplement, (MP):10 mg/day/head probiotic supplemented milk, (MM):50 ml/day/head Myrtus extract supplemented milk, MMP 10 mg/day/head probiotic + 50 ml/day/head Myrtus extract leaf supplemented milk.

[2] Red blood cell count

[3] Mean corpuscular volume

[4] Mean corpuscular hemoglobin

[5] Mean corpuscular hemoglobin concentration

[6] Red cell distribution width

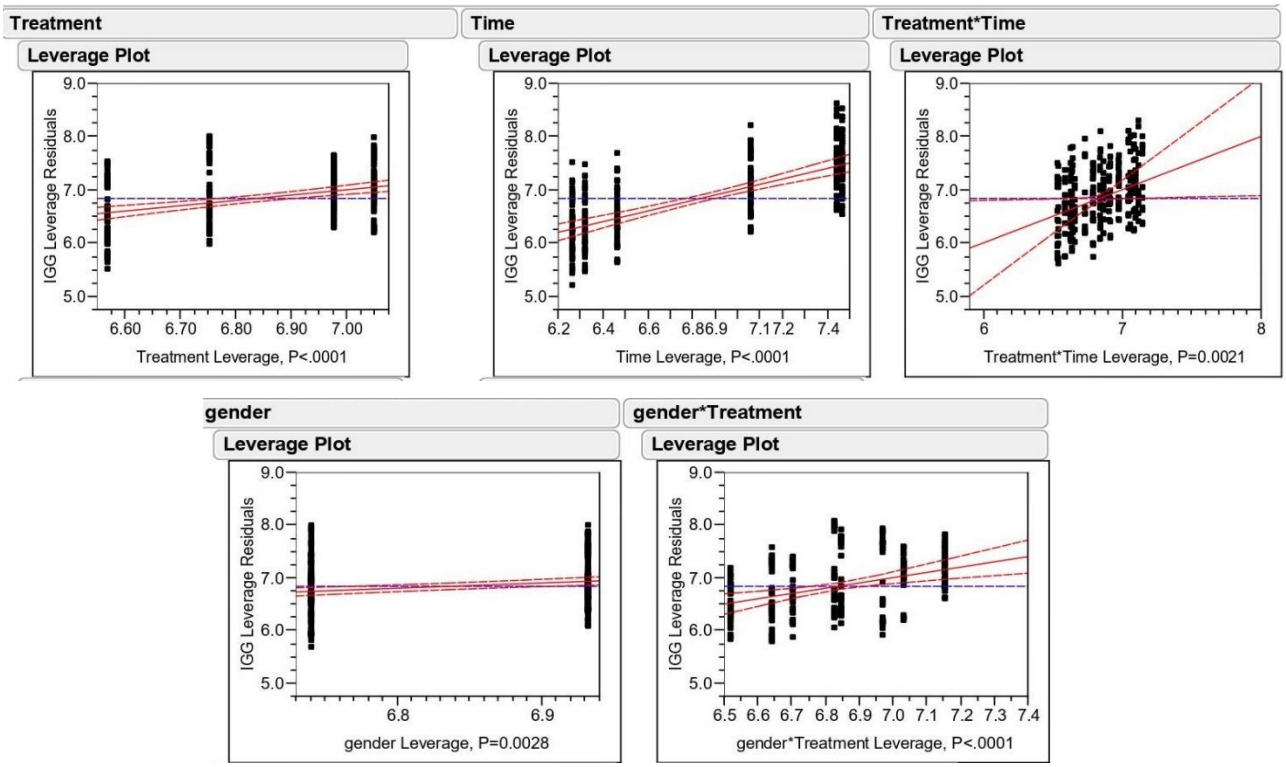
[7] White blood cell count

[8] Standard error of mean

[a,b,c] Values with different superscripts in the same row are significantly different ($P \leq 0.05$) and for tendency declared at ($P < 0.15$)

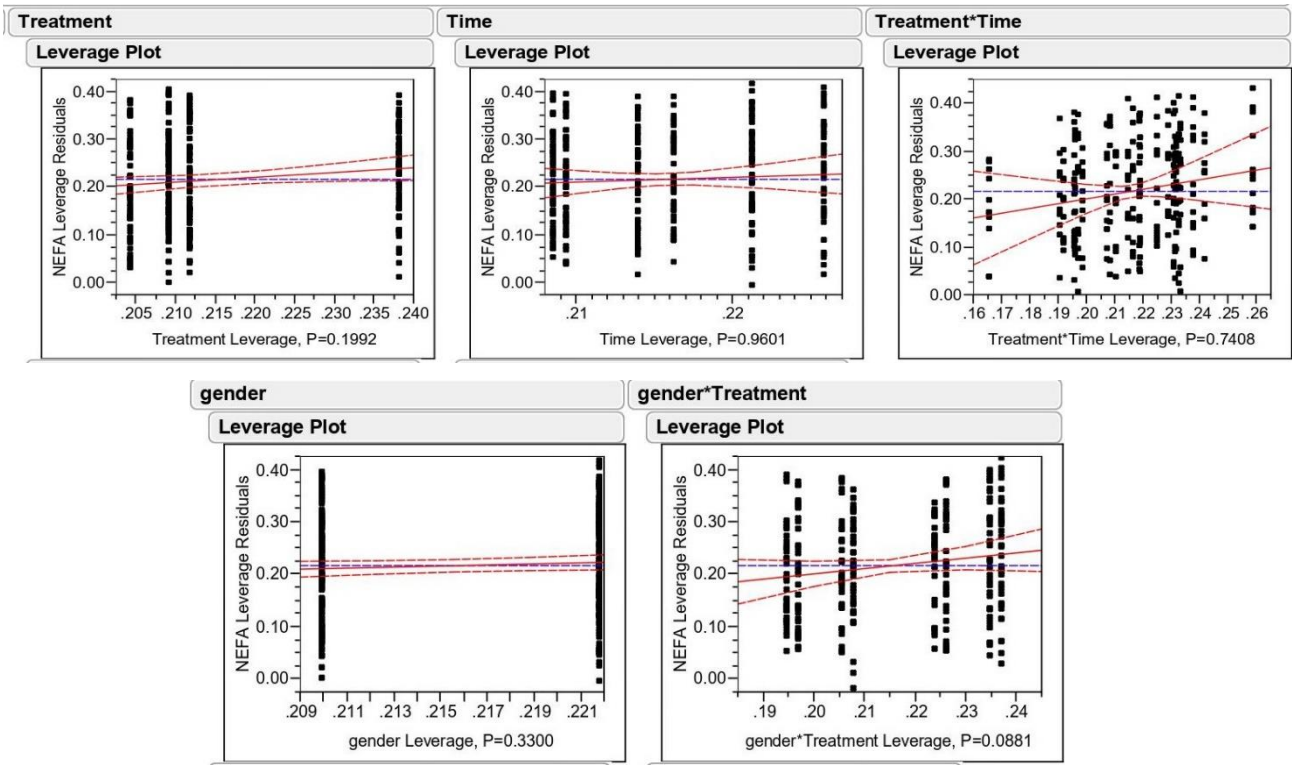
3.4.2 Blood Biochemical Parameters

Graph 3.18: Effects of myrtle plant extract supplementation on Immunoglobulin G



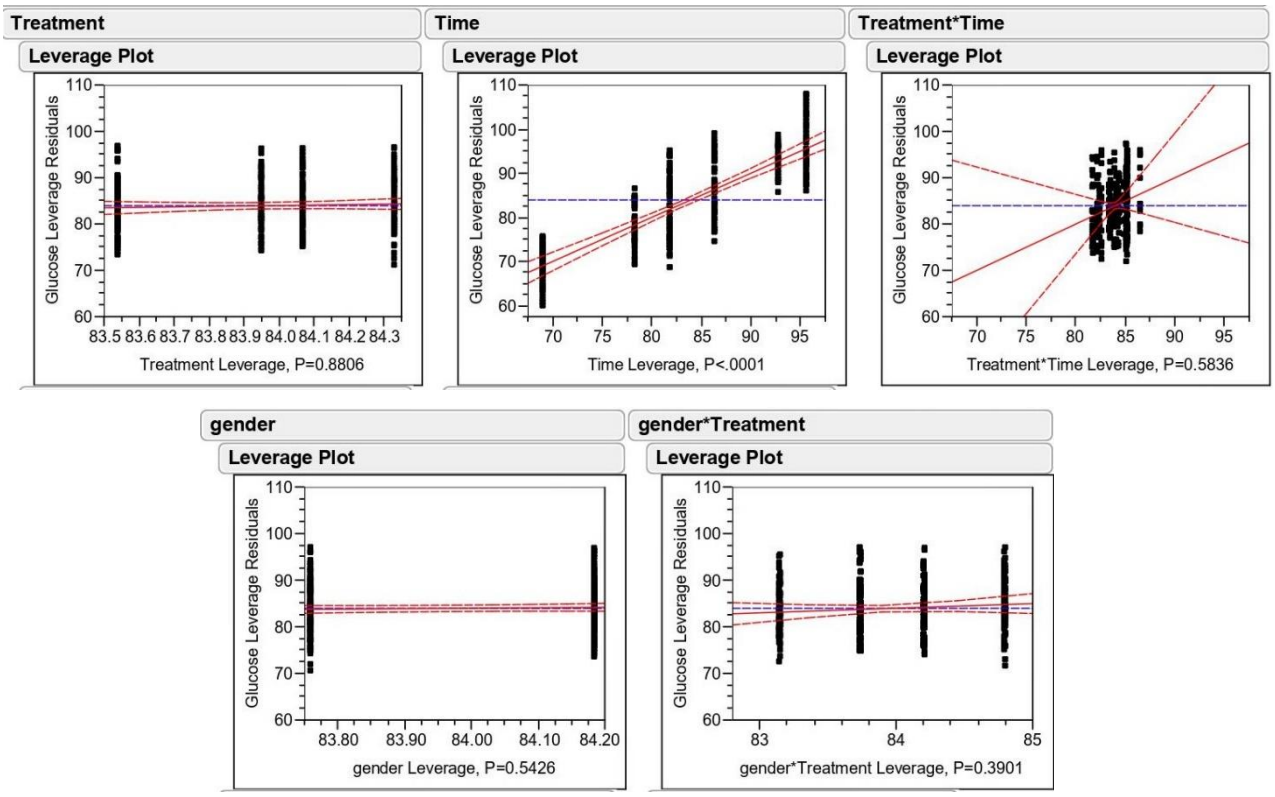
The statistical results showing the effects of supplementation on immunoglobulin G are presented in graph 18. Regarding the hemato-biochemical parameters the supplementation increased the immunity level of the calves which ultimately lead to a strong immunity to counter the calf-hood diseases. The myrtle extract and probiotic supplementation significantly affected both interaction of IgG concentration. The treatment*time interaction was found to be (P=0.0021) whereas the treatment*gender interaction was found to be (P<.0001).

Graph 3.19: Effects of myrtle plant extract supplementation on nonesterified fatty acids



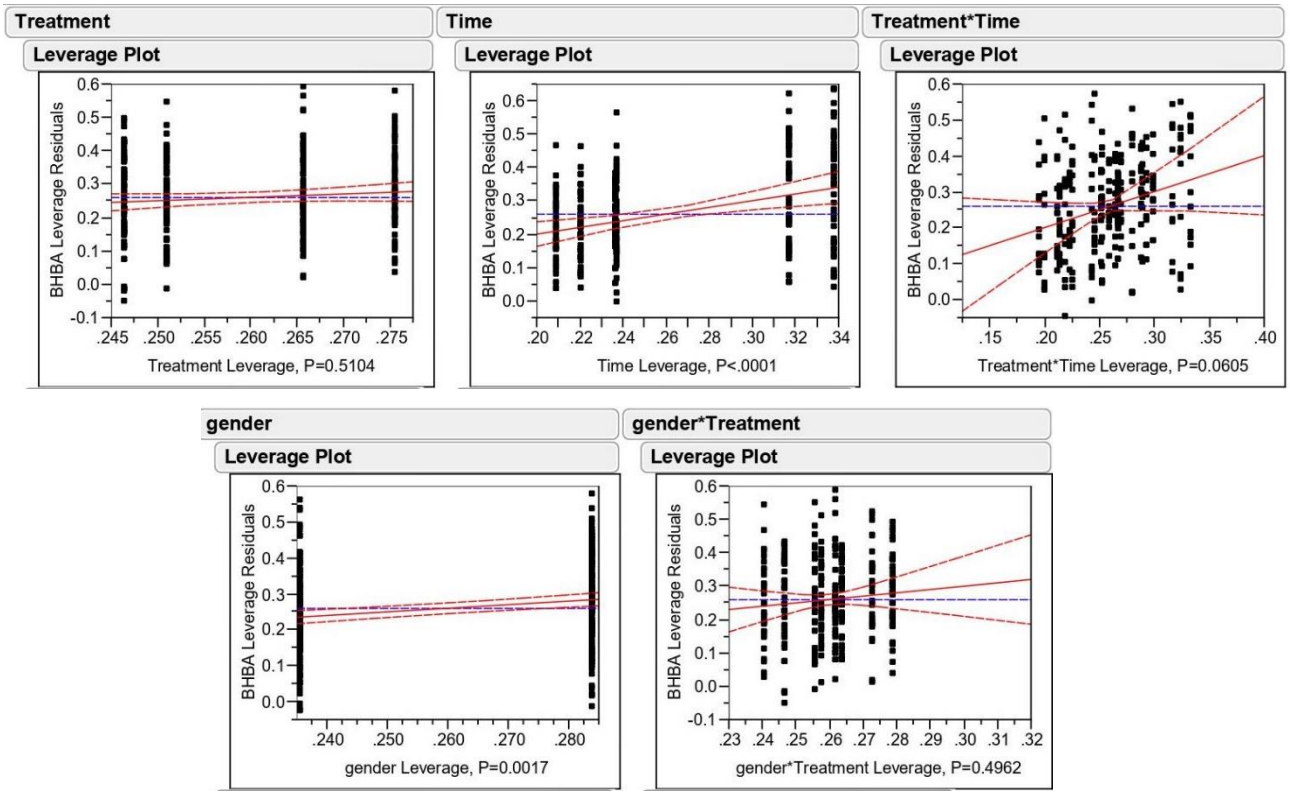
The statistical results showing the effects of supplementation on NEFA are presented in graph 19. Regarding the NEFA concentration the supplementation did not affect treatment*time interaction (P=0.7408) and treatment*gender interaction (P=0.0881).

Graph 3.20: Effects of myrtle plant extract supplementation on Glucose



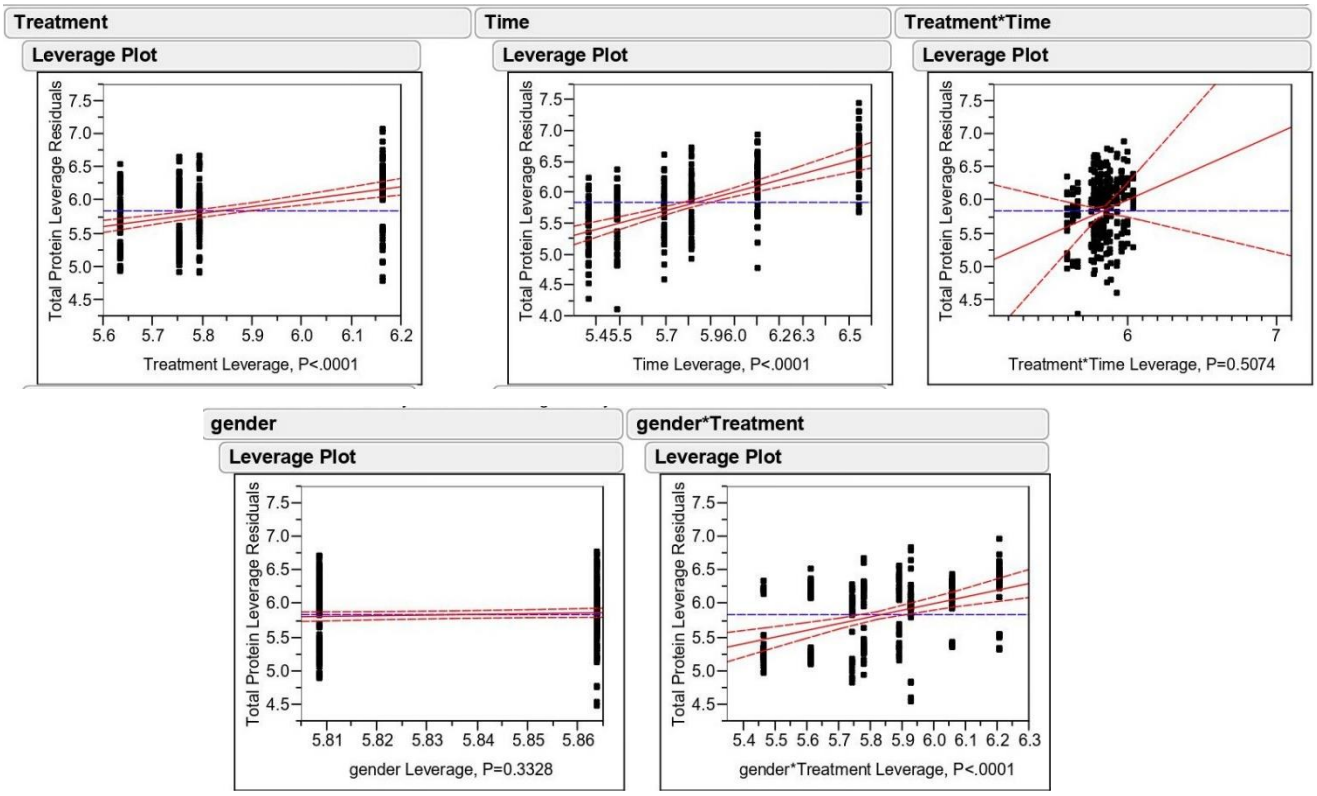
The statistical results showing the effects of supplementation on glucose are presented in graph 20. Regarding the glucose concentration, the supplementation did not affect treatment*time interaction (P=0.5836) and treatment*gender interaction (P=0.3901).

Graph 3.21: Effects of myrtle plant extract supplementation on betahydoxy butyric acids



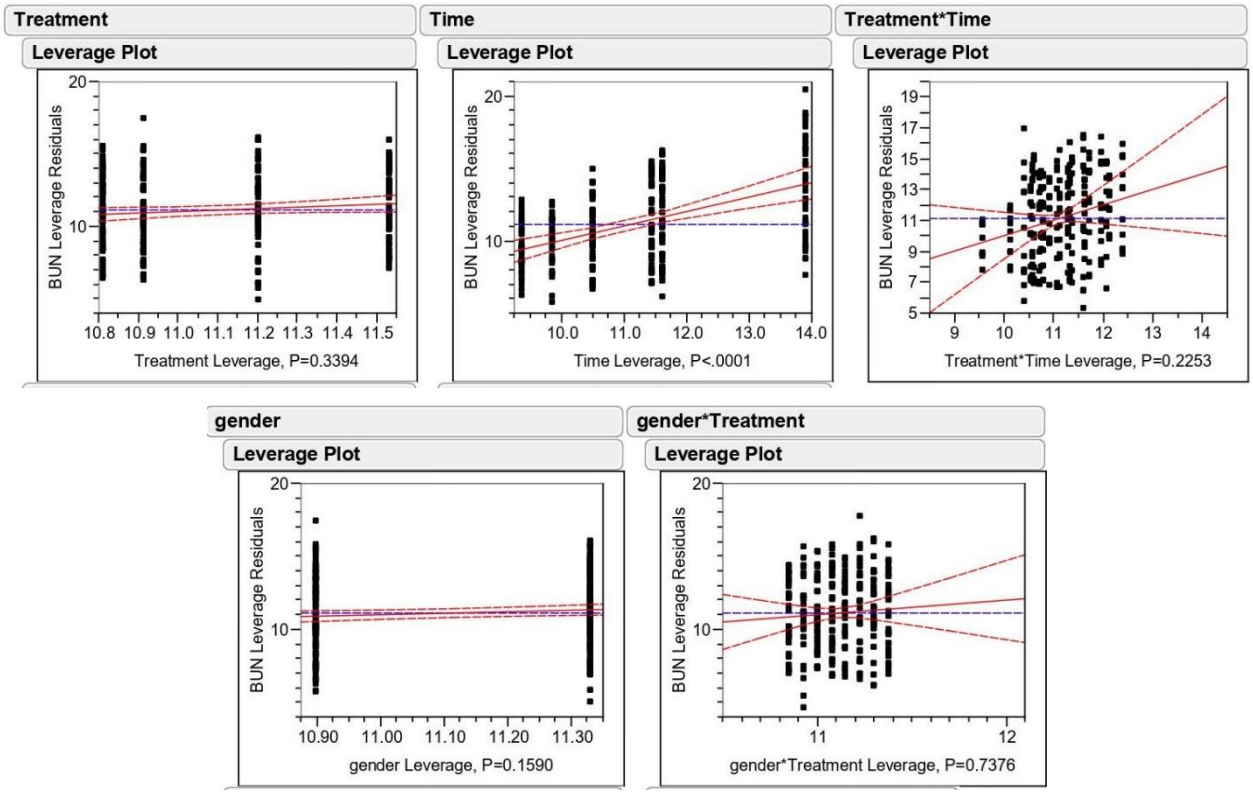
The statistical results showing the effects of supplementation on BHBA are presented in graph 21. Regarding the BHBA concentration the supplementation did not affect treatment*time interaction (P=0.0605) and treatment*gender interaction (P=0.4962).

Graph 3.22: Effects of myrtle plant extract supplementation on Total Protein



The statistical results showing the effects of supplementation on total protein are presented in graph 22. Regarding the total protein concentration, the supplementation did not affect the treatment*time interaction (P=0.5074) whereas the supplementation significantly affected the treatment*gender interaction (P<.0001).

Graph 3.23: Effects of myrtle plant extract supplementation on blood urea nitrogen



The statistical results showing the effects of supplementation on BUN are presented in graph 23. Regarding the effects of supplementation of blood urea nitrogen level, the supplementation did not affect both interactions, the treatment*time interaction was found to be (P=0.2253) whereas the treatment*gender interaction was found to be (P=0.7376).

Table 3.5: Effect of myrtle plant extract and probiotic supplementation on serum biochemical parameters of calves from birth to 75 d of age[1]

	Treatment[4]					P-values				
Item	**Control**	**MP**	**MM**	**MMP**	**SEM[5]**	**Treatment**	**Time**	**Treatment ×Time**	**Gender**	**Treatment× Gender**
Glucose, mg/dl	83.95	83.53	84.06	84.32	0.697	0.8806	<.0001	0.5836	0.5426	0.3901
NEFA[2], mmol/l	0.238	0.209	0.204	0.211	0.012	0.1992	0.9601	0.7408	0.3300	0.0881
BHBA[3], mmol/l	0.246	0.250	0.275	0.265	0.015	0.5104	<.0001	0.0605	0.0017	0.4962
Total protein, g/dl	5.63^{c}	5.75^{bc}	5.79^{b}	6.16^{a}	0.056	<.0001	<.0001	0.5074	0.3328	<.0001
BUN[4], mg/dl	11.20	10.91	10.81	11.53	0.305	0.3394	<.0001	0.2253	0.1590	0.7376
IgG, mg/ml	6.56^{c}	6.97^{a}	7.04^{a}	6.75^{b}	0.063	<.0001	<.0001	0.0021	0.0028	<.0001
AST, U/L	61.60^{a}	60.17^{a}	56.84^{b}	60.22^{a}	1.188	0.0352	<.0001	0.0355	0.0010	0.0006
ALT, U/L	12.19	11.78	12.38	11.51	0.307	0.1814	0.1172	0.1966	0.2170	0.2921
GGT, U/L	153.43	150.76	151.63	151.30	5.487	0.9876	<.0001	0.6196	0.2343	0.0346
Creatinin, mg/dl	0.94	0.99	0.91	0.98	0.068	0.8448	0.0538	0.5793	0.1688	0.9694

[1] Data are represented as least square means, Control: milk without supplement, (MP):10 mg/day/head probiotic supplemented milk, (MM):50 ml/day/head Myrtus extract supplemented milk, MMP 10 mg/day/head probiotic + 50 ml/day/head Myrtus extract leaf supplemented milk.

[2] Non esterified fatty acids

[3] Beta hydroxy butyric acid

[4] Blood urea nitrogen

[5] Standard error of mean

[a,b,c] Values with different superscripts in the same row are significantly different ($P \leq 0.05$) and for tendency declared at $P < 0.15$

3.5 Diarrhea and Pneumonia

Table 3.6: Health status of the groups during the experimental period (birth-75th day)

Groups	Diarrhea	Pneumonia	Number of days with diarrhea	Number of days with Pneumonia
Control	3	2	15	7
MP	1	-	4	-
MM	2	1	9	3
MMP	1	1	3	3

All of the calves experienced same seasonal and environmental conditions and they were housed randomly in hatches of the same shed. The health status of the calves was thoroughly checked with special attention given to diarrhea and pneumonia and if any of the calves suffered from respiratory or digestive disease it was immediately given respective treatment. The treatment included per-orally Kaopektin 80-120 ml/calf, and parenterally through intramuscular i/m route Marbiotic 2 ml/25 kg body weight and Meloxicam 2.5 ml/100 kg body weight for 3 consecutive days. If the calf couldn't recover and become severely dehydrated, then intravenous fluid therapy was given accordingly for effective resuscitation. If the calves loose 8% of their body weight, show signs of depression of central nervous system, either less or no milk intake for atleast 24 hours or have a rectal temperature less than 100 ^{o}F or 38 ^{o}C then fluid therapy becomes necessary. During the trial every day in the morning time before feeding all the calves were physically examined for fecal consistency score, wherein 0 was rated as normal, 1 was rated as semi formed or pasty, 2 was rated as loose but having enough consistency to stay on bedding material and 3 was rated as watery in texture which leaked through the bedding material. According to the physical observations made during the trial it was found that the supplemented calves not only experienced less severity of diarrhea as well as they had less duration spent in diarrhea. Among supplemented groups, MMP group less severe diarrhea and less number of days spent in diarrhea was seen showing positively significant effects of combination of probiotic and phytobiotic supplementation.

Similarly, positively significant effects were observed with reference to pneumonia in the supplemented groups where less severity as well as less number of days spent in pneumonia were observed as compared to the calves of control group. Interestingly no case of pneumonia was observed on MP group.

4. DISCUSSION

4.1 Growth Performance

4.1.1 Effects of Phytogenics on growth performance

The calves of MM group showed increased feed intake, resultantly a statistically significant weight gain was observed as compared to control group which could possibly be due to beneficial and productive effects of phytobioactive compounds by establishing healthy gut environment which resulted in increased absorption of nutrients. Our results, in terms of better feed intake, better feed efficiency and increased weight gain, were aligned with (Miller, et al., 2012) who observed an improved growth production performance of heifers whose milk replacer was supplemented with phytobioactive compounds.

Lemongrass (*Cymbopogon Citrullus*) and peppermint (*Mentha piperita*) improve health and production performance of cattle (Wanapat, et al., 2008; Zmora, et al., 2012). The Chinese herbs *Fructus Ligustri Lucidi*, *Radix Astragali* and *Radix Codonopsis* increased intake of dry matter and organic matter, increased body weight gain, better feed conversion rate, improved health status and milk production of cattle when supplemented 10 g/cow/day (Qiao, et al., 2013). Similarly, cumin seeds (*Cuminum cyminum*) supplemented 200 g/day to cow decrease cholesterol concentrations, improved performance, as well as milk production (Ghafari, M. et al., 2015).

The improved growth performance obtained in our trial is similar to (Seifzadeh, et al., 2016) who found an increased average daily gain and calf starter feed intake of dairy calves by supplementing herbal plant mixture to the dairy calves, similarly our results are also aligned with (Hill, et al., 2007) where the phytobioactive compounds were supplemented to milk and calf milk replacer of Holstein bull calves. The results obtained in our trial, regarding the increased weight gain and biometric measurements (hip height, heart girth, etc), are aligned to the results obtained by (Favaretto, et al., 2020) who supplemented the feed of female lambs with PFA.

A research trial was conducted by (Chaturvedi, et al., 2022) wherein herbal mixture (Haldi, Amla, Tulsi and Arni) was supplemented and its effects were checked on growth performance of Barbari goat kids and the findings, in terms of increased dry matter intake and increased weight gain, were aligned with what we obtained in our trial. The increased growth performance parameters in the

said trial could possibly be due to biomolecules (demethoxycurcumin, tannins, ceryl alcohol, D-mannitol, curcumin, alkaloid, palmitic acid, terpenes, bisdemethoxycurcumin, γ-sitosterol, β-sitosterol, phyllemblin, ascarbic acid, ellagic acid, gallic acid, methyleuganol, euganol, sesquiterpenes, caryophyllene, etc.) (Heibatollah, et al., 2008; Somchit, et al. 2002; Daisy, et al. 2007). The herbs are an effective way to improve health of cattle and quality of animal products however their overall share in total ration is low usually ranges from 0.2 to 2% of dry matter (Caroprese, et al., 2020). These stimulate appetite, increase feed intake and contribute to a better FCR. Kraszewski et al., (2008) found increased feed intake, significantly increased average daily weight gain and improved feed conversion rate when supplemented the feed with 1.0% and 2.0% of herbs: nettle (Urtica), mint (Mentha), thyme (*Thymus vulgaris*), pansy (*Viola tricolor* L.), chamomile (*Matricaria chamomilla*), fennel (*Foeniculum vulgare*), fenugreek (Trigonella) and sage (*Salvia officinalis*).

In a trial by (Klebaniuk, et al., 2014) two types of herbal mixture were supplemented to the diet of young beef cattle, 1st herbal mixture was composed of purple, thyme, cinnamon, oregano, and coneflower and the 2nd herbal mixture was composed of purple, thyme, garlic, licorice, and caraway. The researcher obtained increased ADG, improved FCR and these results were aligned with what we obtained in our trial. The same researcher (Klebaniuk, et al., 2016) performed another trial where he supplemented the diet of beef cattle with dried chopped herbs: licorice (*Glycyrrhiza glabra*), garlic (*Allium sativum* L.), Echinacea (*Echinacea purpurea*), caraway (*Carum carvi*) and thyme (*Thymus vulgaris*) and found increased ADG, increased fat and protein digestibility.

A research trial was performed by (Özçinar, et al., 2023) wherein the myrtle plant extract (extract obtained only from the leaves of myrtle plant) was supplemented to drinking water. The treatment*time interaction was found statistically significant which means that the myrtle plant extract supplementation increased water intake, FCR (p=0.01), put positive effects on production and blood biochemical of Wister albino rats and the results were aligned with our results. It is worth mentioning that the extract used in our trial was extracted from leaves, bark and roots. Similarly, another trial was performed by (Gultepe, et al., 2019) where myrtle plant extract was supplemented, at different proportions, to drinking water given to laying poultry hens to check its effects on different performance parameters. Myrcetin was found to be a major active chemical substance (15.34 mg/L) in myrtle plant extract, checked by liquid chromatography analysis. The

findings of the trial showed increase in feed consumption but did not affect the FCR and both of these are aligned to what observed in our trial.

Similarly, a study by (Varga-Visi, et al., 2023) exhibited statistically significant weight gain when the carvacrol and limonene were supplemented to the feed of sheep and obtained results were aligned with what we obtained in our trial. In another research trial by (Tadayon, et al., 2017) dried orange peel with tannic acid equivalent limonene was supplemented to the diet of fattening lamb which resulted in significant weight gain which was aligned to our trial's results. The effects might be due to phytobioactive compounds.

It was observed in the present trial that the age and intake of starter feed had a direct relationship because with the increase in age, the nutritional reqirements were also increased. The first month of calf's life is of great significance as the intestine of newly born calf is ready to perform at its peak level, the beneficial microbiota residing in the gut is ready to perform at its optimum level but on the other hand, any delay in not supplementing any phytobiotic or probiotic additive to the calf results in drastic consequences. In this context the strategy applied in our trial was aligned with (Timmerman, et al., 2005). The additives can positively affect the growth production performance by not only neutralizing the pathogens but also potentiating the beneficial microbes to perform at their peak level.

4.1.2 Effects of probiotics on growth performance

The probiotics are supplemented as feed additives to the feed of food producing animals to establish a protective microbiota in GIT of calves. In our trial the probiotic was supplemented in whole milk and given to the calves through milk nursing bottles which resulted in increased weight gain, and our findings were aligned with (Timmerman, et al., 2005) who supplemented multispecies probiotic which was composed of various probiotic species of human origin, or a calfspecific probiotic which was comprised of 6 *Lactobacillus* species isolated from calf feces. The gut microbiota secretes active substances which trigger digestive processes and ultimately leads to increased body weightgain. The results obtained in our trial are similar to those obtained by (Jatkauskas and Vrotniakiene. 2010) who found an increased body weight gain by 5.03% (p<0.01) as well as improved FCR when calves were supplemented with Optisaf probiotic @ 10 g/animal/day.

The LAB are beneficial, widely used and are famous for modulating the intestinal environment to get the optimum production performance. Yáñez-Ruiz, et al. (2010) reported that early life of the calf is very crucial and maximum optimization of intestinal microbiota can be achieved by giving probiotic as dietary additive. The findings of our trial were aligned with (Yáñez-Ruiz, et al., 2010) because in our trial we too used newly born calves to get increase in weight gain and increase in feed intake.

The findings obtained in our trial were similar to those documented by (Kumar and Ramana, 2008) who reported increased weight gain in probiotic fed animals, yeast culture diets which supposidely potentiated gut microbiota. Similarly, the results obtained in our study are similar to (Quigley, et al., 1994) who found a positive as well as a direct relation (within unharmful limits) between increased production of volatile fatty acids and dry matter intake.

Not only the results but the biometric measurements (hip height, heart girth, etc.), as reported in our research trial, are in agreement with (Zhang, et al., 2016; Katsoulos, et al., 2017; Renaud, et al., 2019 and Raabis, et al., 2019).

In calf management, pre-weaning is the most important phase of calf's life whereas a good, healthy and productive start of calf's life guarantees significant weight gain, feed intake, better feed conversion rate even in post-weaning phase. However, the neonatal calves are vulnerable and more susceptible to the enteric diseases which results in sub-optimum growth production and special care should be given to save them from calf-hood diseases. The difference in results could be due to health condition, stressful condition experienced by the calves, and exposure to pathogenic microbes. Diet supplemented with high doses of compound probiotics increased the concentrations of immunoglobulins, indicating that the oral administration, with high doses of compound probiotics, may improve the immune systems of calves (Timmerman, et al., 2005). The increasing age and time both significantly affect the feed intake because with the passage of time the calf grows and its energy requirement is increased. In our trial treatment and time had significant effects on feed intake and similar findings were observed by (Baldwin, et al., 2004).

4.2 Digestive Wellness

In the present research trial, diarrhea was confirmed when the fecal score was ≥2 and the condition consecutively remained for 3 or more days. All of the calves experienced same environmental

condition and during the trial relatively less severity and less frequency of diarrhea, good prognosis or recovery from diarrhea and short duration of diarrheic period was observed in the calves of MM and MMP group whichever the calf was affected with diarrhea. On the other hand, severe and more frequent diarrhea and comparatively a longer duration of diarrheic period was observed in the control group (Table 6), corroborated by highest fecal score, was observed during 2-3 weeks of life. The antibiotic treatments were given and sometimes in severe cases, when the condition became worse, fluid therapy was given to the diseased calf of control group. Some calves of the control group suffered from diseases e.g. diarrhea, fever, pneumonia, or cough and they spent more days in diarrheic period whereas calves of the supplemented groups also suffered from digestive or respiratory diseases but they quickly recovered (good prognosis) from these diseases.

The fecal score system is commonly used for predicting diarrhea in young animals. Interestingly, at the first 2 weeks of age, the diarrhea did not differ among the groups, whereas the calves of probiotic and myrtle supplemented groups showed decreased diarrhea at 3rd week of age. The reason may be that in this study, calf starter pellets (from day 7 of age for adaptation and later on day 15 of the age) were given to the calves, it has been proven that substantial development of the forestomach is generally considered obtained within the first 3 to 4 weeks of postnatal life (Renaud, et al., 2020). During the trial it was observed that the calves of supplemented groups not only had less severe diarrhea with good prognosis of recovery but they also spent significantly few days in diarrhea. Interestingly less severity and less duration of diarrhea was seen in the MMP group where combination of probiotic and phytobiotic was given. The findings obtained in our trial are aligned with findings observed in (Gupta, et al., 2020; Bonelli, et al., 2021; Miller. et al., 2012 and Bonelli, et al., 2018). The reason could be due to the anti-diarrheic activities of phytobioactive compounds of the myrtle extract (Gupta, et al., 2020; Bonelli, et al., 2021; Miller. et al., 2012 and Bonelli, et al., 2018). The decreased span of diarrhea in the calves of treatment group proved to be economical in terms of reducing the cost of medication, lactic acidosis, intensive care approach, fluid therapy, and decreased feed intake (Lorenz, et al., 2011).

4.3 Blood parameters

There are two main types of serum proteins, globulin and albumin, present in blood which represents the total amount of protein and collectively these are called total protein or total serum

protein TSP. The albumins are proteins present in blood plasma and synthesized by hepatocytes in the liver and carry almost 75% of osmotic activity in plasma and these are used to transport protein in different metabolic processes. The other part of TSP is globulins which are produced by the immune system of body. The abnormal reference value of TSP in blood plasma is an important diagnostic tool which indicates the disturbance of organism. Immediately after birth, decreased concentration of TSP is found in calves but when the calves take colostrum the concentration of TSP increases (54.5 g/l). It is reported in certain trials that from 5^{th} to 40^{th} day the concentration of TSP slightly decreases whereas after 60^{th} day its concentration increases (Steinhardt & Thielscher, 2000; Knowles, et al., 2000).

The increased level of total protein in blood reflected the enhanced immune response in myrtle supplemented calves of our trial is aligned with (Lakhani, et al., 2019) who performed a trial and supplemented an extract derived from a mixture of 7 plants; mahua seed cake (*Madhuca longifolia*), neem seed cake (*Azadirachta indica*), ajwain seed (*Trachyspermum ammi*), harad (*Terminalia chebula*), fennel seed (*Foeniculum vulgare*), fruit pulp of amla (*Phyllanthus emblica*), and fruit pulp of bahera (*Terminalia bellirica*) at different proportions to the feed of growing buffalo calves. It was also supposed that the high level of serum protein might be attributed to the essential oils of extract obtained from the mixture of those 7 plants which exerted a positive impact on the ruminal microbial protein synthesis. In a trial by (Kholif, et al., 2012) the goats are fed ginger, garlic or cinnamon oil and in another trial by (Olafadehan, 2011) the goats are fed tannin-rich forage, in both of the trials an increased level of total protein is observed which is aligned with the increased level of total protein observed in our trial. Similar to our results an increased level of TP was found in a trial conducted by (Abd El-Hack, et al., 2019) wherein 0.5 gm of mixture of red and black pepper was supplemented to the diet of rabbits. An elevated level of globulin was found in steers which were fed pepper, supposedly the increased level of pepper might be due to pro-inflammatory response. As already suggested, the increase in globulins in steers who consumed pepper may be related to a pro-inflammatory response. Furthermore, it was noted through literature that pepper extract puts an inhibitory effect on the production of inflammatory cytokine and the proliferation of cells without being toxic resultantly inflammatory protein expression is suppressed (Hazekawa et al., 2017).

Diet supplemented with phytobiotic compounds increased concentrations of Ig, indicating that phytobiotics may improve the immune systems of calves. The immune responses are due to the

establishment and enhancement of innate and adapted immune system which is an indication of a sound health and an enhanced immune system of animal. Similarly, another trial was performed by (Gultepe, et al., 2019) where myrtle plant extract was supplemented, at different proportions, to drinking water given to laying poultry hens to check its effects on immunological parameters. The liquid chromatography analysis was performed and the myrcetin was found to be a major active chemical substance (15.34 mg/L) in myrtle plant extract which might have affected the immunity status. The results showed a statistically significant amount of IgG (p<0.05) in 5% MPE supplemented group and the findings are aligned to what observed in our trial.

A trial by (Jiang, et al., 2022) wherein 3 Chinese herbs were supplemented and its effect was checked on serum biochemical and immunity parameters of yak calves. The IgG level was increased in yak calves who consumed extracts of 3 Chinese herbs; *Angelica sinensis*, *Codonopsis pilosula* and *Glycyrrhiza uralensis***.** The response might be due to active ingredients or bioactivities of these herbs. The herb *Angelica sinensis* comprised of important active ingredients e.g. volatile oils, polysaccharides, phthalides, and organic acids which have the potential to give protection against cardiovascular diseases and myocardial infarction, atherosclerosis, arrhythmia, and enhance immune responses (Wei, et al., 2016). Similarly, *Codonopsis pilosula* is composed of alkynes, alkaloids, flavonoids, saccharides, and terpenes, which give protection to GIT, enhance the circulation, potentiate the immune response (Li, et al., 2018). On the other hand, *Glycyrrhiza uralensis* contains important phenolic compounds (chalcones, coumarins and flavonoids) and triterpenoid saponin, it is used as harmonizing agent with anti-inflammatory, antioxidant, anti-proliferative activities and chemo-preventive (Rahman and Sultana, 2007).

5. CONCLUSION

The feed intake, growth performance and immunity of calves were significantly affected by MM supplementation during the experiment. MM decreased the incidence of diarrhea in calves before weaning. There was a significant improvement in growth performance, a reduction in calf diarrhea and an overall improvement in serum immunity compared to the control group. Therefore, we conclude and recommend supplementing the milk of dairy calves with myrtle extract as a domestic product alone or a mixture of myrtle extract and probiotics during the pre-weaning period.

6. RECOMMENDATION AND SUGGESTIONS

In this trial myrtle plant extract and probiotic were supplemented to the daily milk given to the calves, however, it is suggested that the myrtle plant extract should be supplemented by making slurry of the feed (in various proportions and ratios of myrtle extract and feed) to check its effects on different nutritional parameters and health status of calves. In this context some further trials should be conducted to check the biological significance of the active compound obtained from myrtle plant by supplementing it either through milk replacer or feed slurry.

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