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REVIEW

Review: Do Horses Receive Optimum Amounts of Glutamine in Their Diets?

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ABSTRACT

In some species of growing mammals glutamine is an essential amino acid that, if inadequate in the diet, is needed for normal growth and development. It is thus sometimes considered to be a conditionally essential amino acid in some species. A review of studies that have measured L-glutamine concentrations ([glutamine]) in horses demonstrates that plasma [glutamine] has routinely been reported to be much lower (~330 μmol/L) than in other mammals (> 600 μmol/L). Plasma [glutamine] represents the balance between intestinal transport into the blood after hepatic first pass, tissue synthesis and cellular extraction. The hypothesis is proposed that sustained low plasma [glutamine] represents a chronic state of sub-optimal glutamine intake and glutamine synthesis that does not meet the requirements for optimum health. While this may be without serious consequence in feral and sedentary horses, there is evidence that provision of supplemental dietary glutamine ameliorates a number of health consequences, particularly in horses with elevated metabolic demands. The present review provides evidence that glutamine is very important (and perhaps essential) for intestinal epithelial cells in mammals including horses, that horses with low plasma [glutamine] represents a sub-optimal state of well-being, and that horses supplemented with glutamine exhibit physiological and health benefits.

1. Introduction

Glutamine is an important metabolite involved in gluconeogenesis, lipolysis, antioxidant defense, the production of nitric oxide, the secretion of peptides (e.g. glucagon-like peptide 1, GLP-1), neuromediators, the regulation of cell growth, regulation of cellular function and cell / tissue regeneration [1-5]. The importance of glutamine in numerous cellular processes, and the fact that plasma concentrations ([glutamine]) appears to be quite low (by ~50%) in most horses studied compared to other mammals, suggests that dietary glutamine may be inadequate in many horses. Unfortunately, the dietary requirement for glutamine has been considered only with respect to protein synthesis. However dietary glutamine requirements also need to be considered with respect to its numerous other functions in the body. For example, in other species inadequate glutamine is associated with increased incidence and severity of respiratory disease [6], brain disorders [7], type 1 diabetes [5], slowed muscle glycogen synthesis [4], impaired gut morphology and health [8-11] and impaired immune health [12,13].

The purposes of this review are to report the concentrations of glutamine in equine plasma and muscle in studies

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published in the past 50 years and to use a comparative physiology approach to demonstrate that dietary glutamine ingestion appears to be low in most horses studied, and perhaps in the general horse population. Some of the equine studies reviewed herein have already suggested that horses may benefit from dietary glutamine supplementation. Many amino acids, including glutamine, are ingested in proteins and peptides as well as free amino acids as part of the normal diet. Some amino acids (glutamine for example) can be synthesized within the body, while others cannot. The ability to synthesize certain amino acids varies by species and developmental stage. In horses, except for lysine, the dietary requirements for amino acids have not been defined. The recommended protein intake [14] may provide adequate amino acids for protein synthesis related to growth and development, however recent studies have reported that the dietary supply of some amino acids may be in inadequate. With respect to protein synthesis, these amino acids now include lysine [15], threonine [15,16] and methionine [17].

Under normal conditions, the body appears to attempt to meet glutamine demand primarily by *de novo* synthesis (Figure 1) from endogenous glutamate and branched-chain amino acids within tissues such as skeletal muscle [18-21], liver [18] and placenta [19]. Plasma [glutamine] is therefore the result primarily of tissue glutamine synthesis and tissue glutamine extraction [3,20], and secondarily of dietary intake especially in the first few hours after meal ingestion [22-24]. During periods of increased metabolism (exercise, gestation, lactation) a state of protein catabolism often occurs [18,19,22,25]. The sustained or increased tissue glutamine demand and metabolism during these states are associated with reduced plasma [glutamine] (Table 1). Under these conditions glutamine has been termed a "conditionally essential" amino acid [2,3,8,12,26,27].

2. Importance of glutamine

The term glutamine will be used to refer specifically to L-glutamine, one of twenty amino acids that are used to build proteins under the guidance of the genetic codes. Glutamine exists in two zwitterionic forms, L-glutamine and D-glutamine. Because both the amino and carboxyl groups are attached to the first (alpha, α) carbon, glutamine is classified as an α-amino acid. Glutamine is also neutral, i.e., it possesses no electrical charge. Glutamine is additionally the amide of glutamic acid, another naturally occurring amino acid, and is involved in various metabolic activities including the formation of glutamate, and the synthesis of proteins, nucleotides and amino sugars.

The total amount of glutamine in the body is approximately 400 g in adult horses [18]. Glutamine plays important roles within intestinal tissues and skeletal muscle, and the body as a whole, including regulation of cellular gene expression, neuronal excitability, protein turnover, cellular metabolism, immunity and acid-base balance [27-29]. The amino acids glutamine and glutamate make up 10-20% of dietary protein, and both are extensively metabolized in the small intestine of most mammals. Watford [30] asserts that, with normal levels of dietary intake (5 - 10 g of glutamine daily for humans), there is no net small-intestinal absorption of glutamine or glutamate into the blood, such that body's glutamine pool results from de novo synthesis, primarily within skeletal muscle. In various mammals, including humans, about 20% of dietary glutamine may end up in the systemic circulation, but this is dependent on the amount of glutamine ingested and the metabolic state. Therefore the high requirement for glutamine by intestinal enterocyte and immune cells result in considerably less glutamine entering the systemic circulation than what is ingested.

There is now good evidence that glutamine, and some other non-essential amino acids, are not synthesized in sufficient amounts during periods of increased metabolic rate to support fetal development, neonatal growth, growth during lactation and as needed to maintain optimal vascular health, intestinal health and immune function in adult animals [27]. These amino acids have therefore been re-classified as "conditionally essential" [2,31] or "functional" amino acids (FAAs) because of their inadequacy in the diet, particularly in young and gestating mammals and during normal periods of increased metabolism such as during exercise and physical training. Inadequate intake of FAAs leads to functional deficits due to impairments in the regulation of key metabolic pathways involved in health, growth, development, reproduction and lactation [10,28,30,32]. Fürst et al. [33] characterized glutamine as a "conditionally indispensable amino acid during stress", where stress is the commonly-used physiological term to describe the normal physiological responses that result in elevated metabolism.

Glutamine should be considered to be a "conditionally essential" α-amino acid that is nutritionally important for many animals including horses and humans, particularly during periods in which the metabolic state of the animal is normally elevated, such as during normal exercise, gestation, lactation, growth and development [2]. Supplementation of FAAs such as glutamine, in amounts adequate to meet nutritional and metabolic requirements, has been proposed [2,8] as a nutritional strategy to maintain or improve health, growth and development and to prevent diseases. Xi et al. [28] reported that adequate, high concentrations of intracellular and extracellular glutamine are

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associated with marked reductions in infection, sepsis, severe burn, cancer, and other pathologies. For example, oral glutamine supplementation in healthy humans performing moderate intensity exercise prevented the exercise-induced increase in intestinal permeability [34], thus maintaining integrity of the intestinal - immune system during periods of elevated metabolism.

Skeletal muscle is the major tissue that synthesizes glutamine by virtue of its mass in the body. The enzyme glutamine synthetase catalyzes the synthesis of glutamine from ammonia and glutamate. Mammalian skeletal muscle comprises approximately 40% of lean body mass, and intramuscular glutamine serves as a regulator of the anabolic state of this tissue. In nourished mammals, skeletal muscle releases glutamine into the circulation at a rate of 40 - 60 mmol/h [32,35]. When dietary intake of glutamine is low (e.g., as a result of typical horse forage) circulating concentrations of glutamine are low, and about 13-60% of that found when dietary glutamine is high (Table 1). The circulatory system provides a means of transporting glutamine to those cells that require it and that are not capable of synthesizing adequate amounts to meet their demands (Figure 1).

The liver, like skeletal muscle, both synthesizes and consumes glutamine. The enzymes for each process are compartmentalized to different hepatic cell systems [30]. The liver normally produces a small amount of glutamine and plays a role in fine-tuning plasma [glutamine].

In the kidneys, glutamine serves as the major substrate for ammoniagenesis, the process of removing nitrogen from the body, and in whole-body acid-base balance [30]. The glomeruli filter glutamine, but it is nearly completely resorbed by the renal tubules. During extended periods of increased metabolism (prolonged exercise, physical training, pregnancy, lactation, some diseases, including some cancers) resulting in net whole-body catabolism, there is a large increase in immune and intestinal cellular glutamine utilization, as well as increased hepatic extraction where glutamine is used for acute phase protein synthesis and glucose production. Exocrine signals acting on skeletal muscle result in a net proteolysis within muscle cells and increased net glutamine synthesis. This is often accompanied by decreased intestinal glutamine utilization [30], with consequent impairment of intestinal and immune function [28].

Glutamine is converted to glutamate in the brain and serves important roles in neurotransmitter regulation. In particular, glutamate regulates the neurotransmitter gamma-aminobutyric acid (GABA), which is required for brain functioning and mental activity. Glutamine newly synthesized from ammonia and glutamate by astrocytes within the brain is extracted by neurons. Enzymes then hydrolyze the intracellular glutamine back to glutamate, some of which is decarboxylated to produce GABA, or transaminated to aspartate [10].

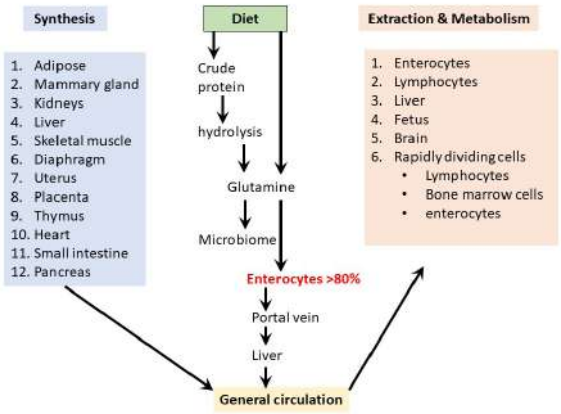

Figure 1. An overview of the tissues involved in the synthesis, transport and metabolism of glutamine. Most circulating glutamine is produced by glutamine-synthesizing tissues. Within the small intestine, greater than 80% of glutamine is extracted by and metabolized by enterocytes. Very little ingested glutamine enters the general circulation

Glutamine is essential for lymphocyte proliferation (which are unable to synthesize glutamine) and other rapidly dividing cells, such as gut mucosa and bone marrow (mesenchymal) stem cells; inadequate glutamine supply is associated with impaired function in these cellular systems [12,13,35]. High rates of leukocyte (particularly lymphocyte) glutamine extraction and utilization has led to the classification of glutamine as an immunostimulant.

Glutamine serves as a metabolic precursor for other important amino acids such as arginine, citrulline and proline. Within the intestinal system (splanchnic bed), unoxidized glutamine and proline serve as important precursors for citrulline synthesis, which is then converted to arginine in the kidney [36]. Arginine is a semi-indispensable amino acid in neonates and serves numerous metabolic roles in young and adult mammals [3,12].

Glutamine also plays important roles in whole-body biochemical and energy regulation. For example, it serves as a substrate for several amidotransferases that synthesize purines, pyrimidines, NAD, glucosamine and asparagine [30,32]. Most of the body's glutamine is hydrolyzed to glutamate and ammonia via the action of glutaminase. Glutamate can, in turn, be converted into glutathione, proline, ornithine and arginine, it can also be catalyzed to produce glucose, or it can be oxidized to produce ATP. The carbon is excreted as carbon dioxide and the nitrogen is excreted

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as ammonia and urea.

Amino acids, through a large variety of inhibitory mechanisms and signaling pathways, act to regulate gene expression in numerous cell types within the body. Transcription factors mediate these effects, including specific regulatory sequences, such as amino acid response elements that are sensitive to changes in amino acid concentration [37]. In particular, glutamine, at appropriate concentrations, enhances numerous cell functions by activating various transcription factors. Some of the better-understood functions include the inflammatory response, cell proliferation, cell differentiation and survival, and several metabolic functions.

In summary, Ruth and Field [38] identified the following metabolic functions for glutamine:

(1) serves as a precursor and energy substrate for immune and epithelial cells;

(2) is important for intestinal development and function and for maintaining the integrity of the gut barrier, the structure of the intestinal mucosa, and redox homeostasis;

(3) supports proliferative rates and reduces enterocyte apoptosis;

(4) protects against pathogenic bacterial damage to intestinal structure and barrier function;

(5) lowers inflammatory response and increases immunoregulatory cytokine production; and improves the proliferative responses and numbers of intestinal immune cells.

2.1 The Small Intestine - Immune System Relationship

The present understanding of the intimate relationship between the gut (entire gastro-intestinal system) and the immune system has recently been presented by Ruth and Field [38] and Miron and Cristea [39]. There is considerable agreement amongst mammalian studies across species, and it is highly likely that the key elements observed in other mammals are transferable to horses:

(1) the intestine is the main site of nutrient absorption and amino acid metabolism, and the gut-associated lymphoid tissue (GALT) is also the largest immune system organ in the body.

(2) the enterocytes play multiple roles with respect to immune function and maintenance of immune health, including protection against oral pathogens, inducing oral tolerance to food stuffs, and maintaining a healthy interaction with commensal bacteria.

(3) the enterocytes also maintain barrier function between luminal contents (external environment) and the internal environment of the body (also reference [40]). This barrier function is dependent on dietary glutamine availability (also reference [34]).

(4) dietary amino acids (in particular, glutamine, glutamate, arginine, and perhaps methionine, cysteine and threonine) are essential to optimize the enterocytes' and intestinal immune cells' immune functions (i.e., dendritic cells, beta cells, macrophages, T cells). Each has unique properties essential for maintaining the intestine's integrity, growth and function, and for regulating local tissue and organ immune responses.

Glutamine supplementation has been shown to be effective in maintaining a normal intestinal barrier against pathogens and preserve mucosal integrity [8,34,38,39,41-43]. Using rodent models of intestinal mucosal obstruction and injury (similar to an equine obstructive small intestinal "colic"), glutamine supplementation prevented the large increases in intestinal permeability and bacterial translocation seen in non-supplemented animals [42,43].

3. Are Horses Deficient in Glutamine?

Compared to other amino acids, the concentrations of glutamine are relatively high in plasma (0.30 - 2.0 mmol/L depending on species and stage of development) and skeletal muscle (up to 3 mmol/L or approximately 60 mmol/kg wet weight). These concentrations provide an indication of the importance of glutamine within the body. With low to normal amounts of dietary glutamine, up to 100% of the glutamine ingested with protein is utilized by cells of the small intestine. In this typical situation, none of the dietary glutamine enters the systemic circulation [30]. Instead, plasma concentrations of glutamine are maintained by de novo synthesis from metabolic precursors. In this sense glutamine is considered to be non-essential.

In horses, the pre-feeding or fasting tissue glutamine concentrations reported in most studies (Table 1) are considerably lower than those reported in well-fed, healthy humans [44] (500 - 700 µmol/L) and rats [45,46] (700 - 1,000 µmol/L), and horses [47] (900 - 1,000 µmol/L). The fact that research horses receiving a near-optimum diet have average plasma [glutamine] ranging from 880 to 1,020 µmol/L [47] lends further support to the theory that dietary glutamine may be physiologically limiting in many horses.

3.1 Horses at Rest

In adult horses a number of studies published over a nearly 50-year period have reported an average plasma glutamine concentration of about 300 µmol/L (Table 1). The lowest values appear to be 150 µmol/L [48] and the highest values about 1,000 µmol/L [47]. In foals peak values occur at 2 weeks, and decline to values seen in adults by time of

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Table 1. Fasting or pre-feeding plasma and muscle glutamine concentrations in horses

Study	Breed (number)	Age	Tissue	Concentration[1]
Johnson and Hart 1974 [72]	Mixed (12)	adult	Plasma	293 ± 21
Rogers et al. 1984 [22]	QH mares (10)	10 - 12 months gestation and 1st 3 weeks lactation	Mare plasma Mare plasma Mare plasma Foal plasma	300 (12 w prepartum) 600 (1-2 d postpartum) 511 (3 w postpartum) 736 (3 w postpartum)
Russell et al. 1986 [73]	QH (6)	22 months	Plasma	15[2]
Miller & Lawrence 1988 [64]	QH (6)	adult	Plasma Plasma	493 ± 48 (low protein) 393 ± 18 (high protein)
Miller-Graber et al. 1990 [98]	unknown (4)	adult	Muscle	~450 ± 40
Jahn et al. 1991 [54]	TB (24)	2 - 4 years	Plasma	227 ± 14
Duckworth et al. 1992 [75]	Mixed (7)	adult	Plasma	572 ± 24
Silver et al. 1994 [50]	Pony mares (12)	At 235 - 308 days of gestation	Maternal plasma Fetal plasma Maternal at 36 h fast	370 ± 20 510 ± 50 247 ± 31
Zicker et al. 1994 [25]	Mixed (6)	45 - 47 week gestation mares	Facial artery Uterine vein Umbilical artery Umbilical vein	268 ± 10 270 ± 14 682 ± 42 723 ± 27
King & Suleiman 1998 [69]	TB (6)	adult	Plasma	367 ± 19
Routledge et al. 1999 [53]	Mixed (19)	6 - 12 years	Plasma	470 ± 15 310 ± 20
Robson et al. 2003 [55]	Endurance traineda	9.4 ± 2.2 years	Plasma	279 ± 16
Harris et al. 2006 [66]	TB (6)	5 - 9 years	Plasma	320 ± 30 280 ± 20 250 ± 25
Hackl et al. 2006 [24]	Various (10) 40 day trial	9 - 14 months	Plasma	304 ± 9 (start study) 345 ± 15 (end study)
Hackl et al. 2009 [62]	SB trotters (36)	2 - 10 years	Plasma	385 ± 16*
Manso Filho et al 2008 [18]	SB (3)	1., 10 & 30 years	Skeletal muscle	3,000 to 5,000
Manso Filho et al. 2008 [19]	SB (8)	Pregnant mares	Plasma Plasma Plasma Skeletal muscle	290 ± 20, 7 w prepartum 510 ± 20, parturition 290 ± 15, 8 w postpart. 7,000 ± 700 (all times)
Manso Filho et al. 2009 [20]	SB (8)	Mares at birth	Amniotic fluid Placenta	310 ± 25 2,800 ± 2,100
Urschel et al. 2010 [48]	TB (6)	4 - 8 years	Plasma	149 ± 21 155 ± 20
Van den Hoven et al. 2010 [21]	SB (10)	2.5 - 6 years	Plasma Skeletal muscle Skeletal muscle	403 (209 - 663) 1,800 ± 1,100 2,500 ± 1,200
Westermann et al. 2011 [61]	SB (10)	20 + 2 months	Plasma	392 ± 62*
Nostell et al. 2012 [63]	SB trotters (12)	4 - 9 years	Plasma	255 ± 30
Urschel et al. 2012 [47]	Arabian (12)	9 - 22 years	Plasma	1,060 ± 60
Peters et al. 2013 [60]	Warmblood mares (6)	12 + 3 years	Plasma	281 ± 40
Tanner et al. 2014 [16]	Not stated (6)	6 mo weanlings	Plasma	561 ± 24
Mastellar et al. 2016 [23]	TB (6 & 6)	176 + 30 days Adult mares	Plasma Plasma	610 ± 20 424 ± 19
Mastellar et al. 2016 [97]	TB (6)	1 year	Plasma	223 ± 42 ~1,000 ± 110

Notes: Values are mean ± standard error. [1] Plasma: μmol/L; Muscle, placenta: μmol/kg wet weight. [2] reported values in this paper are 10-fold less than likely, which would make this ~150 μmol/L. * Significant decrease with exercise. QH = quarter horse; SB = Standardbred; TB = Thoroughbred.

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weaning [49]. Skeletal muscle [glutamine] is typically an order of magnitude greater than that of plasma, but also declines rapidly (within 1 month) in foals [49].

Manso Filho et al. [49] examined muscle and plasma [glutamine] during the first year of life in Standardbred foals. Glutamine was one of the most abundant free α-amino acids in skeletal muscle at birth. The concentration at 7 days declined by more than 50% by 6 months with no change thereafter. The initially high glutamine concentrations can in part be explained by provision of glutamine in mother's milk, and occurring at a time of elevated glutamine synthesis in mares that doubles plasma [glutamine] at the time of parturition [19,22] and mirrors elevated umbilical plasma vs maternal plasma [glutamine] [25,50]. As solid foods are introduced and the horse is weaned, then plasma [glutamine] decreases to reflect the balance between tissue requirements (related to metabolic activity) and dietary intake. One interpretation is that low plasma glutamine concentrations reflects a high demand for glutamine, thus the prevalent plasma [glutamine] of ~300 μmol/L may indicate a state of suboptimal glutamine synthesis and provision.

The fact that so many equine studies report relatively low plasma glutamine concentrations, while there is evidence that plasma glutamine in horses can be as high as normally seen in humans, raises the possibility of chronic hypoglutaminemia in the general horse population. It is proposed that a chronic hypoglutaminemia would be due to inadequate dietary supply of glutamine [2,27] combined with inadequate rates of endogenous glutamine synthesis from metabolic precursors. From these results it is concluded that the low tissue glutamine concentrations reported in most equine studies reflect typical equine diets that are low in dietary sources of glutamine such that dietary supplementation of glutamine may be appropriate.

3.2 Horses with Elevated Metabolic Needs

3.2.1 Pregnancy and Lactation

During pregnancy, the fetus extracts glutamine from the placental circulation [49] and umbilical [25] plasma [glutamine] is more than double that of mares' general circulation [25]. There is a large increase (approx. doubling) of plasma [glutamine] between 2 weeks pre-partum and the first few days post-partum [22]. Lactation is also very metabolically demanding period: the mammary glands extract glutamine during lactation and [glutamine] is abundant in the milk of lactating mares [19], although. After peaking at 1 - 2 weeks of lactation, by 3 months of lactation in mares' plasma and milk [glutamine] had decreased by more than 50% which, together with loss of lean body mass, is indicative of a mild catabolic state [49]. The authors concluded that the decrease in circulating [glutamine] during lactation, when large amounts of glutamine are being extracted by the mammary gland, "means that glutamine availability for maternal organs, such as the small intestine and immune cells, may be limiting as lactation proceeds".

Despite the capacity of key tissues, predominantly skeletal muscle, to synthesize glutamine, these data, though limited, suggest that increased dietary glutamine intake would be needed in order to maintain adequate (> 500 μmol/L) plasma [glutamine]. Plasma [glutamine] averaged 360 μmol/L pre-fasting in seven lactating mares, but fell to 247 μmol/L after 36 h of fasting and recovered to only 318 μmol/L 6 h after additional feeding [50]. While fetal plasma [glutamine] were nearly double those of their mares [22,49], the fetal plasma [glutamine] similarly decreased after fasting [22]. This, in part, reflects the high requirement of the developing fetus for glutamine, despite the high capacity of the placenta to synthesize glutamine [20].

Dietary composition also has pronounced effects on plasma glutamine concentrations in the transition (peripartum through to beginning of lactation) mare; the dietary provision of even non-glutamine-containing supplements added to forage more than doubled plasma [glutamine] [22]. This provides evidence that the provision of other nutrients to glutamine-producing cells and tissues, increases the production and release of glutamine by these cells / tissues into the blood at a crucial time when glutamine is in high demand by other cells / tissues. It is also an indication that whole body glutamine demands have not been adequately met prior to provision of additional nutrients. In horses in a catabolic state, dietary supplementation of glutamine can help minimize or prevent the catabolic state and be used to maintain steady [glutamine] essential for intestinal and immune function and health [51].

Consistent with the equine studies cited above, Wu [52] considers dietary glutamine to be "substantially inadequate" to meet the requirements for protein synthesis in extra-intestinal tissues in growing pigs. By extension, this author infers that such is the case for mammals during periods of elevated metabolism (exercise, lactation, active growth and development [27]). A typical diet does not provide sufficient arginine, proline, aspartate, glutamate, glutamine, or glycine for optimum protein accretion in growing pigs [52].

3.2.2 Exercise

Exercise, whether of long-term low intensity or short-term high intensity, imposes significant increases in cellular and whole body metabolism often associated with increased

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skeletal muscle proteolysis [35,53]. As a result the intramuscular and plasma concentrations of some amino acids and ammonia increase. Plasma [glutamine] also rises in part as a result of proteolysis and in part due to an increased requirement to detoxify ammonia [54].

In horses, humans and rodents, moderate to high intensity or duration exercise results in immune function suppression [35,55-57]. Exercise typically, but not always [54], depresses plasma glutamine [35,57], but this result also needs to be considered in the context of intensity of exercise and timing of post-exercising sampling. Glutamine supplementation also enhances the immune response to intense exercise, effects that appear to be mediated by intestinal/immune system interaction [35,58,59]. Glutamine supplementation prevents the increase in intestinal permeability that occurs during moderate intensity exercise [34]. Neutrophils, which comprise 50-60% of the total leukocyte count, elicit some of the beneficial effects seen with glutamine supplementation [56].

Supplemented, exercise-conditioned rats performed one hour of exercise at 85% of peak VO_2 [56]. In one group of rats, glutamine was supplemented by oral gavage one hour before exercise. Compared to the control group that did not receive glutamine, the supplemented rats' neutrophils had significantly increased phagocytic capacity. The supplemented rats also showed a smaller decrease in nitric oxide production than normally seen with intense exercise and higher production of reactive oxygen species.

In the equine hindlimb, glutamine appears not to be utilized by muscle as a fuel source during exercise [60] and only contributed to about 1.3% of the VO_2 at rest. Post-exercise, average hindlimb venous plasma [glutamine] was slightly greater than arterial. This may indicate net synthesis and release by muscle in order to meet glutamine demand of other tissues in the body, but definitive research remains to be performed.

In horses performing a constant speed, 20-minute duration, high intensity (about 80% of peak VO_2) exercise test [61], and with high-intensity maximal speed exercise [62], plasma [glutamine] decreased significantly immediately after exercise and did not recover.

Horses performing a very high intensity (about 115% of peak VO_2) exercise test had decreased plasma [glutamine] 5 minutes after exercise, and a significant recovery peaking at 30 to 60 minutes, followed by a gradual decline to typical post-prandial steady-state values [53]. These authors and Jahn et al. [54] attributed the post-exercise glutamine increase to ammonia detoxification associated with the increased intramuscular ammonia production. Similar results were reported with horses completing high intensity field exercise testing [63].

The decrease in plasma [glutamine] associated with relatively high intensity exercise contrasts with the increase seen by Jahn et al. [54] and during constant-speed moderate-intensity exercise [64] and was of a magnitude similar to the osmotic loss of plasma fluid (decrease in plasma volume [65] suggesting no net addition or loss of glutamine during this type of exercise. Plasma [glutamine] returned gradually to pre-exercise values over a 30-minute period.

When Harris et al. [66] supplemented dietary glutamine (single feeding and 10 days of supplementation at 30 and 60 mg/kg body mass; equal to about 15 and 30 grams, respectively) in athletically-worked horses, they found that even this relatively low amount of supplementation nearly doubled plasma [glutamine]. They concluded that increasing plasma [glutamine] through the diet has "benefit in the athletically worked horse with lowered plasma glutamine concentrations". A recent study in horses supplemented with a dietary protein / amino acid mixture within the first hour of completing high intensity exercise concluded that supplementation directly after training decreases post-exercise skeletal muscle proteolysis [67].

When Matsui et al. [68] infused radio-labeled phenylalanine (for calculating amino acid kinetics in horse muscle) they showed that intravenous administration of an amino acid mixture shortly after heavy exercise decreased the rate of muscle proteolysis and increased the rate of protein synthesis in the hind limb. van den Hoven et al. [21] reported that oral administration of amino acids to horses within 1 hour after exercise increased the intramuscular amino acid concentrations. Using exercise trained horses, van den Hoven et al. [21] supplemented the diet with amino acids for 6 weeks. High intensity exercise resulted in a 16% decrease in muscle [glutamine], followed by a 30% increase in muscle [glutamine] 4 hours after completion of exercise. This was associated with a 25% increase in post-exercise plasma [glutamine] when the amino acid supplement was offered during the first hour post-exercise. By 18 hours after exercise, plasma and muscle values had returned to pre-exercise baseline values. Both of these studies indicate a benefit, if not a need, for supplementary dietary glutamine, as well as some other amino acids, as a result of exercise, even in horses receiving daily supplements of amino acids.

Robson et al. [55] examined the effects of long-term endurance exercise (80 km endurance race) on plasma [glutamine] and immune function parameters. Pre-race plasma [glutamine] were low (279 $\pm$ 16 μmol/L). That there was no decrease immediately post-race, one hour post-race, and one-day and three-days post-race may be attributed to these very low starting values. In the post-race period, the horses experienced decreased neutrophil oxidative burst

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activity and up to a three-fold decrease in circulating lymphocytes that was not fully recovered by three days post-race (impaired immune response). In these athletic horses, it appears that low plasma [glutamine] contributed to the severity of the observed immune depression. The results also indicate that these endurance horses did not receive adequate dietary glutamine.

A 16-week, regular exercise training program for Thoroughbred horses [69], and 4 to 16-week training periods of varying intensities using Standardbred horses [61], had no effect on plasma [glutamine] pre- versus post-training, which remained between 300 and 500 μmol/L. There do not appear to be studies that have examined the effect of standard race-training programs on glutamine concentrations and tissue stores.

A viral challenge (equine influenza virus) of six horses resulted in a gradual and progressive ~30% decrease in plasma [glutamine] over a six-day period, and [glutamine] remained depressed for at least an additional eight days [53]. The study authors attributed this result to an increased requirement for glutamine by immune system cells. A sustained decrease in circulating [glutamine] was suggested to impair the horses' ability to mount an effective immune response.

In summary, the evidence provided in this section indicates that dietary glutamine, and perhaps other amino acids that influence tissue glutamine synthesis, are not provided in adequate amounts to maintain optimum health. Intestinal health and immune health appear to be the best studied with respect to deficiency of glutamine, but effects on other systems may come to light as research continues.

4. Dietary and Supplemented Glutamine

The main sources of glutamine in the equine diet are plant proteins from forage and from supplemented grains [14]. The crude protein (CP) content of forages ranges from very low - with timothy at about 8% and alfalfa as high as 25% [70,71]. For horses, there is no glutamine recommended dietary allowance (RDA). The NRC [14] states that the daily protein requirement is 0.49 - 0.68 g /kg body mass (compared to 0.6 - 0.8 g/kg in humans). For horses in light to moderate work, this translates to 250 g of CP per day for a 450 kg horse, which provides up to 40 g of glutamine daily based on the typical proportions of amino acid in equine diets [14]. The recommendation increases to approximately 320 g CP/day for 450 kg horses in heavy work, which translates to 51 g of glutamine daily. A recent study using isotopically labeled amino acids compared two protein-supplemented diets in weanling horses, with horses receiving either 3.1 g or 4.1 g CP/kg body weight/day [16]. Compared to horses receiving the lower amount of crude protein, horses receiving the higher amount of crude protein showed time-dependent increases in plasma amino acid concentrations, including glutamine, and that these horses had a higher rate of whole body net protein synthesis. Tanner et al. [16] concluded that, in the lower CP group, provision of at least one amino acid potentially limited the rate at which protein synthesis occurred.

In horses, as with other animals that consume dietary protein, plasma amino acid concentrations depend on feed composition, time of blood sampling relative to meals and tissue amino acid turnover [24,66,72,73]. When Miller and Lawrence [64] fed diets containing 12.9% versus 18.5% crude protein for two weeks, plasma [glutamine] was actually greater on the control diet (493 $\pm$ 18 μmol/L) compared to two weeks on the high protein diet (393 $\pm$ 18 μmol/L). While the amino acid profile of the diets had not been determined one interpretation of these results is that provision of additional amino acids in the high protein diet may have resulted in increased demand for glutamine or reduced synthesis of glutamine.

In response to the consumption of single meals, plasma [glutamine] typically increases with a peak occurring three to five hours after feeding [22-24,53,73]. The increase in plasma [glutamine] can be explained by absorption of glutamine into the blood from the intestinal system (mainly small intestine) and/or from de novo synthesis of glutamine. This distinction must be made because studies that have directly examined intestinal glutamine transport have reported little to no glutamine entry into the circulation [74-76]. The lion's share of intestinal (luminal) glutamine is taken up by enterocytes [76] and metabolized [74]. Of the glutamine that does enter the portal circulation, the portal drained viscera extracts about two-thirds of the circulating glutamine [75]. Thus ingested glutamine poorly accounts for the observed plasma glutamine concentrations. At stated by Manso Filho et al. [18] the great majority of glutamine within the body of the horse must be synthesized de novo through the action of glutamine synthetase. This conclusion is supported by the inverse relationship between tissue glutamine concentration and glutamine synthase protein expression in horses [18].

In the post-feeding period if food is withheld, then between 32 and 48 hours after feeding plasma [glutamine] rises somewhat for several hours [73]. This sustained elevation during the fasting period reflects release of synthesized glutamine into the circulation and indicates the importance of maintaining elevated plasma [glutamine].

In all mammals, and, indeed, most vertebrates, the primary functions of the small intestine are to absorb both water and low molecular weight nutrients exiting from the stomach. These nutrients include mono- and di-saccha-

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rides (i.e. glucose, fructose), amino acids and dipeptides, free fatty acids, monovalent and divalent cations and anions (electrolytes), vitamins and trace minerals. These functions in the horse are similar to that of other mammals [77-82].

Many nutrients are transported into the blood by the intestinal system via the portal circulation (i.e., the blood supply to the liver from the intestinal system). In contrast, many amino acids enter the small intestine but do not enter the portal circulation, and thus do not make their way to the rest of the body. The intestines use 20% of the extracted amino acids for intestinal mucosal protein synthesis and the remainder for many other metabolic processes, including providing oxidative energy. For example, two-thirds of the glutamine, one-third of the proline and nearly all of the glutamate and aspartate are catabolized within swine small intestines rather than absorbed into the circulatory system [52]. Enterocytes are the major site of glutamine extraction and oxidative ATP production, particularly the absorptive columnar epithelial cells of the small intestine [30]. While one-third of glutamine not extracted by epithelial cells lining the intestinal lumen enters the portal circulation, cells of the small intestine also absorb some of this glutamine from the arterial circulation. This basal membrane route of glutamine entry into enterocytes appears to be important for the maintenance of gut health and immune function. In contrast to glutamine, most EAAs entering the small intestine are not extensively metabolized within enterocytes.

Enterocytes along the length of the equine small intestine are well endowed with a variety of transporters for neutral (e.g. glutamine) and cationic amino acids [83], although there are some modest differences in the amino acid transporter rates and affinities between horses and omnivores [84]. The large capacity for glutamine transport results in the majority of ingested glutamine being transported into enterocytes along the entire length of the intestinal system, with most of the uptake occurring from the small intestine [76,85].

Salloum et al. [76] studied the transport of glutamine into equine luminal enterocytes isolated from the jejunum. Similar to that of other mammals, the system B sodium-dependent transporter accounted for about 80% of the total transport. In a complementary study using anesthetized adult horses, Duckworth et al. [75] measured the capacity of the small intestine to extract glutamine from the arterial circulation. The extraction of glutamine by the equine jejunum in vivo more than doubled when the arterial concentration of glutamine was increased by bolus infusion, and jejunal extraction of glutamine was greater than that in the large intestine.

5. Safety of Supplemented Glutamine

Only one peer-reviewed scientific study has examined safety of orally supplemented glutamine (very low amount) in horses and no adverse events were reported [86]. Therefore the peer-reviewed scientific literature on other species is used to provide an indication of safety in horses, keeping in mind the similarities between horses and other mammals with respect to small-intestinal and immune-system functions. The capacity of the intestinal system, skeletal muscle, liver and kidneys to extract glutamine and glutamate is high, and Bertolo and Burrin [36] found that diets rich in glutamine or glutamate have little effect on circulating concentrations and low potential for toxicity.

In humans there is no defined Tolerable Upper Intake Level for dietary protein and 35% percent of total energy intake from protein is considered safe [87]. Within this context, and based on the absence of adverse effects, the Observed Safety Limit of glutamine supplementation (i.e., the highest amount one can consume that will not cause side effects) was identified to be 14 g/d (20 mg/kg body mass) in supplemental form above normal food intake in normal healthy adults [88]. This equates to 100 g/day for a 500 kg horse. Higher dietary intake levels have been tested in humans and shown to be well tolerated (for review see Wischmeyer [89], Watford [30]. In humans and other animals, ingestion of approximately 0.75 g glutamine/kg body mass (in the range of 40 - 60 grams per day) may increase plasma ammonia concentrations above the tolerated safety limit [90]. When orally supplemented below 40 gram per day in humans (~0.5 g/kg body mass) no adverse effects were reported [91,92]. Holecek [92] reported that intake levels of 40 grams or more consumed per day, glutamine: (1) may impair amino acid transport and distribution among tissues because it competes with other amino acids for transport systems [76], such that individuals with reduced kidney function should carefully consider glutamine requirements; (2) may impair synthesis of endogenous glutamine and enhance glutamate and ammonia production; (3) may impair ammonia detoxification; and (4) may result in an abnormal balance of amino acids in the body. Intake levels as high as 2.0 g/kg body mass in rats caused only an approximate 30% increase in brain striatal glutamine concentrations and only a 13% increase in striatal fluid GABA concentrations [93]. Fifty human subjects aged 17 - 65 years old ingested a carbohydrate/glutamine (50 grams of glutamine) supplement less than 20 hours prior to elective bowel surgery and no adverse effects were observed. The authors concluded that this amount of acute glutamine supplementation was safe during pre-op-

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erative "fasting" and subsequent surgery [94]. Elderly men and women (69 ± 8.8 years) ingesting 0.5 g/kg supplemental glutamine had no increase in plasma ammonia levels, although these subjects did have increased serum urea and creatinine (within the normal range) that were deemed not clinically relevant [95]. In critically ill children, several studies have shown that glutamine supplementation was safe and did not cause toxic levels of ammonia or glutamate that could be suggestive of neurotoxicity (reviewed by Albrecht et al. [29]. Single oral doses of glutamine of 20 - 22 g/kg, 8 - 11 g/kg, and 19 g/kg were lethal in mice, rats, and rabbits, respectively [96].

Based on these data, and considering the relevant similarities between species with respect to glutamine metabolism, it can be concluded that supplementing dietary glutamine at up to 0.4 g/kg body mass above that provided in normal diets (13% crude protein) is safe for horses with healthy renal function in the long term. It is also indicated that short periods (one to two days) using dosages at high as 0.6 g/kg body mass may be safe when dealing with horses that have exceptionally high glutamine demands (late gestation, lactation, post-surgery, and after very stressful exercise or transport).

6. Summary and Conclusions

In summary, tissue glutamine concentrations are lower in most horses studied than in other mammals, reflecting diet composition, tissue glutamine requirements and possible dietary inadequacy. During normal periods of increased metabolic activity (lactation, growth and development of young, exercise and training), glutamine requirements are increased and glutamine availability appears to often not be adequate to meet requirements for optimum health. Dietary provision of glutamine has utility in minimizing or preventing catabolic states associated with periods of increased metabolic rate (exercise, lactation). Diets deficient in glutamine do not provide sufficient glutamine to enterocytes and or other body systems. Dietary glutamine supplementation resulted in significant increases in horses' systemic glutamine concentrations. Glutamine supplementation can help athletic horses increase plasma glutamine concentrations. All athletic horses tested have had low plasma glutamine concentrations, typically half that of well-fed healthy horses and healthy humans. A sustained decrease in plasma glutamine impairs a horse's ability to mount an effective immune response. During periods of inadequate dietary intake, inadequate tissue concentrations of glutamine are associated with impaired health, growth, development, intestinal function and immunity.

In conclusion, it is proposed that supplementary dietary glutamine will support intestinal cell nutrition, the immune system, and the general health of horses. Consideration of the numerous benefits afforded by adequate dietary intake of glutamine has led to the animal feeds and supplements industries to develop, produce and market numerous glutamine-containing dietary supplements for horses, cattle, sheep, humans, swine, poultry and fish. As stated by Wu and coworkers [8,27,28,52] supplementing conventional diets with glutamine can optimize growth in young animals and help maintain health in animals and humans.

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ARTICLE

Epidemiology and Antibiotic Susceptibility Profile of Methicillin Sensitive *Staphylococcus aureus* among Livestock and Pet Animals

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ABSTRACT

Staphylococcus aureus is an important zoonotic pathogen that is responsible for a variety of infectious diseases in humans and animals. The present study was designed to check the prevalence and antimicrobial resistance of MSSA from three different animal origins (bovine, caprine and pet). A total of n= 450 samples (150 each source) were collected from bovine, caprine and pets. Collected samples were subjected to *S. aureus* identification by microbiological examination and confirmed *S. aureus* isolates were put to oxacillin disk diffusion test to declare them MSSA. The MSSA confirmed isolates were subjected to various antibiotics for susceptibility profiling using Kirby Bauer Disk Diffusion test. The present study found higher prevalence of MSSA from caprine origin (goat 83.33%) as compared to pet (cat 69.33%; dog 65.33%) and bovine origin (buffalo 26.66%; cattle 31.66%). The *in-vitro* findings of current study revealed oxytetracycline and gentamicin presented 100% efficacy against MSSA of all origins while the vancomycin presented >35%, >40% and > 65% resistance against MSSA isolated from bovine, caprine and pet origin respectively. However, ciprofloxacin was equally effective (50%) against MSSA from buffalo and cattle while >80% efficacy was noted against MSSA from cat and dogs. Linezolid and amoxicillin+ clavulanic acid were 77.78% and 66.67% sensitive to MSSA isolates from caprine milk. The present study found higher prevalence of MSSA from bovine, caprine and pet isolates with diversified pattern of susceptibility of different antibiotics from all sources.

1. Introduction

Animal human bond has very primitive history as this interaction helps the psychological and physical wellbeing of the person [1]. Animals have a powerful impact in human history as they had served as cavalry horses, sentry dogs, carrier pigeons, and unit mascots, or unofficially as a Soldier's battle companion [2]. Animals can be used as a powerful tool to cope psychological challenges and as a therapeutic mo-

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dality or as an adaptive intervention to facilitate positive rehabilitation outcomes [3]. Pet introduction in human life as a natural extension helps to cope psychological challenges, unleash overburdened healthy life activities, least visits to doctors and part of recreative leisure [4]. A goat is generally entitled as "cow of poor man" [5].

Staphylococcus aureus is an important zoonotic pathogen that is responsible for a variety of infectious diseases of both cadre [6]. *S. aureus* has emerged as superbug of animal and human by compromising health and economy [7,8]. Studies report it to be second most common etiology accounting to 17 million annual human deaths [9]. About 25-40% of healthy people have *S. aureus* on their skin and nasal cavity [10]. *S. aureus* is a commensal bacteria as well as opportunistic pathogen and capable of colonizing at different sites in a variety of animals species and humans [11]. *S. aureus* has been screened from various sites of animals including the skin, ear, nasal cavity and anal region [12]. Almost 25 % of humans also harbor *S. aureus* in the nasal cavity [13]. Dog nasal cavity is the most frequently known site for colonization when cultures from various sites were processed [14]. *S. aureus* strains have been isolated from animal origin foods like poultry, pork, beef, milk and dairy products [15]especially those expressing a multidrug resistance (MDR).

Public health is exposed to a bitter challenge of antibiotic resistance by the pathogens which results in treatment failure, longer disease course, increased costs of treatment, more morbidity and mortality. [16]. Resistance is the means by which organism responds to changing environment for survival. [17]. *S. aureus* has been assigned to be multidrug resistant. Four resistance mechanisms can be observed in *S. aureus* including trapping of drug, alteration in drug target, drug inactivation by enzymatic pathways and transmembrane efflux pump activation [18]. Methicillin resistant *S. aureus* strains have been designated as emerging pathogen in livestock and companion animals. Hospital acquired MRSA and community associated MRSA are limited to humans only, no cross-infection chances are there. But livestock occupational personals may have infections with animal originated MRSA.[19] Devastating resistance pattern of MRSA of human as well as animal origin against commonly used antibiotics has been reported [20-22]successful strategies to combat MRSA need strong and coordinated efforts from both, the human and the veterinary field according to the "One Health" concept. Not only MRSA strains are point of concern for such resistive behavior, methicillin sensitive *S. aureus* (MSSA) strains are also on the way to adopt the same resistance mechanism against commonly used antibiotics [23-26]presenting a major and constantly changing clinical challenge. Therefore, present study was designed to check the prevalence and antibiotic resistance pattern of methicillin sensitive *S. aureus* of pets, caprine, and bovine origin.

2. Materials and Methods

2.1 Sample Collection

The sampling was done from pets (dogs, cats) brought to the clinic and dairy farms, located in and around district Faisalabad, Punjab, Pakistan. Total of n=450 samples were collected from all sources having n= 150 from each source using convenient sampling technique [27]. A total of n= 150 were collected from pets (n=75 dog, n=75 cat), n= 150 from bovine (n=90 buffalo, n= 60 cattle) and n= 150 from caprine (goat). Sterile swabs dipped in phosphate buffered saline (PBS) were used for sampling from nose and ear of dogs and cats while milk samples were collected after cleaning the teats, discarding a few streams of milk and scrubbing the teat ends with cotton balls moistened with 70% alcohol. The collected samples were shifted to the laboratory of Institute of Microbiology, University of Agriculture Faisalabad maintaining cold chain (4˚C) for further processing.

2.2 Identification and Confirmation of *Staphylococcus aureus*

Collected samples were cultured on blood agar and overnight incubation was done at 37˚C, for 24 hours for best possible retrieval of *S. aureus* and further culturing was done on Mannitol Salt Agar (MSA) following same incubation conditions. The confirmation of *S. aureus* based on pooled information from culture characteristics, microscopic evaluation and biochemical tests following guidelines of Bergey's Manual of Determinative Bacteriology [28].

2.3 Identification of Methicillin Sensitive *S. aureus* (MSSA)

S. aureus confirmed isolates from all sources were put to oxacillin disk diffusion test following the guidelines of Clinical Laboratory and Standard Institute [29]. Briefly, fresh cultures of *S. aureus* adjusted at 1.5×10^8 CFU/ml were swabbed on Muller Hinton Agar (MHA) plates whereas antibiotic discs were aseptically placed at equal distances from each other. Incubation was given at 37˚C for 24 hours and zones of inhibition were measured and

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compared with standards of CLSI to declare resistant, sensitive or intermediate strains.

2.4 *In-vitro* Efficacy of Various Antibiotics against Methicillin Sensitive *Staphylococcus aureus*

Methicillin sensitive *S. aureus* isolates from all sources were put to in-vitro antibiotic susceptibility testing against various antibiotics i.e. vancomycin (30µg), ampicillin (10µg), chloramphenicol (10µg), enoxacin (10µg), amoxicillin (10µg), fusidic acid (10µg), amoxicillin + clavulanic acid (20µg) ciprofloxacin (10µg), oxytetracycline (30µg), gentamicin (30µg), amikacin (30µg), and trimethoprim-sulfamethoxazole (25µg) using Kirby Bauer disc diffusion test [30]. Fresh culture adjusted at 1.5×10^8 CFU were swabbed on Muller Hinton Agar whereas antibiotic discs were aseptically placed at equal distances from each other following the guidelines of Clinical Laboratory Institute [29]. Incubation was given at 37°C for 24 hours and zones of inhibition were measured by Vernier calliper in millimetres [30] and compared with standards of CLSI to declare resistant, sensitive or intermediate strains [29].

2.5 Statistical Analysis

Prevalence was determined by using formula described by [27].

$$\text{Prevalence}(\%) = \frac{\text{No. of infected Animal(n)}}{\text{Total no. of sampled Animals(N)}} \times 100$$

The descriptive statistics was applied for estimation of antibacterial assays.

3. Results

3.1 Prevalence of Methicillin Sensitive *Staphylococcus aureus* (MSSA) Isolated from Bovine, Caprine and Pet Origins

The present study found 59.78% (269/450) overall prevalence of MSSA isolated from bovine, caprine, and pets. However, higher prevalence of MSSA was found from caprine origin (goat 83.33%) as compared to pet (cat 69.33%; dog 65.33%) and bovine origin (buffalo 26.66%; cattle 31.66%) (Table 1). The prevalence of MSSA was noted to be higher 69.33% from cats as compared to dogs 65.33%. Similarly, MSSA percentage was noted higher 31.33% as compared to 26.66% from cattle and buffalo origin respectively. The study found significant difference ($p< 0.05$) among all cadre of MSSA origin.

Table 1. Prevalence of methicillin sensitive *Staphylococcus aureus* isolated from bovine, caprine and pet origins

Sample origin	Species	Total	Positive	Percentage	C.I	*p-value*
Bovine	Buffalo	90	24	26.66%	18.63-36.62	0.000
	Cattle	60	19	31.66%	21.31-44.24	
Caprine	Goat	150	125	83.33%	76.55-88.45	
Pet	Dog	75	49	65.33%	54.05-75.11	
	Cat	75	52	69.33%	58.17-78.61	
	Total	450	269	59.78%	55.19-64.21	-

3.2 *In-vitro* Therapeutics Efficacy of Various Antibiotics against Methicillin Sensitive *Staphylococcus aureus* Isolated from Bovine Milk

The findings of present study revealed Oxytetracycline and Gentamicin presented 100%, Ciprofloxacin showed 50% efficacies against MSSA isolated from both cattle and buffalo milk. However, Trimethoprim-Sulphmethoxazole and Vancomycin showed 30% and 23.08% efficacy against MSSA obtained from buffalo while no efficacy was noted against MSSA of cattle origin. The present study found 100% resistance and intermediate variants of fusidic acid and enoxacin against MSSA of buffalo and cattle origin respectively. Amikacin efficacy was increased from 33.33% to 100% against MSSA isolated from buffalo milk as compared to cattle milk. Antibiotic susceptibility profile of various antibiotics against MSSA of bovine origin was observed during current study (Table 2).

Table 2. *In-vitro* therapeutics efficacy of various antibiotics against methicillin sensitive *Staphylococcus aureus* isolated from bovine milk

Antibiotic Name	Potency	Buffalo			Cattle		
		R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Enoxacin	10ug	0.000	100	0.000	0.000	100	0.000
Amikacin	30ug	66.67	0.000	33.33	0.000	0.000	100
Fusidic acid	10ug	100	0.000	0.000	100	0.000	0.000
Ciprofloxacin	5ug	50.00	0.000	50.00	50.00	0.000	50.00
Vancomycin	30ug	38.45	38.47	23.08	66.67	33.33	0.000
Oxytetracycline	30ug	0.000	0.000	100	0.000	0.000	100
Trimethoprim-Sulphmethoxazole	25ug	70.00	20.00	30.00	50.00	50.00	0.000
Gentamicin	30ug	0.000	0.000	100	0.000	0.000	100

Note: R= Resistant, I= Intermediate, S= Sensitive

3.3 *In-vitro* Therapeutics Efficacy of Various Antibiotics against Methicillin Sensitive *Staphylococcus aureus* Isolated from Goat Milk

The *in-vitro* findings of current study reported that MSSA

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isolates were 100% sensitive to Gentamicin and Oxytetracycline followed by Trimethoprim + Sulphamethoxazole and Cefoxitin (88.89%), Linezolid 77.78%, Chloramphenicol and Amoxicillin+Clavulanic acid 66.67%, Amoxicillin 44.44%, and Vancomycin 22.22%. However, it presented higher resistance to Vancomycin and Amoxicillin 44.44%, followed by 22.22% to Amoxicillin+ Clavulanic acid, 11.11% to Chloramphenicol. However, intermediate type of response was shown against Vancomycin 33.33%, Chloramphenicol and Linezolid 22.22%, followed by Amoxicillin, Cefoxitin, Amoxicillin, Trimethoprim + Sulphamethoxazole and Amoxicillin+Clavulanic acid 11.11%. Antibiotic susceptibility profile of various antibiotics against MSSA of caprine origin was observed during this study (Table 3).

Table 3. *In-vitro* therapeutics efficacy of various antibiotics against methicillin sensitive *Staphylococcus aureus* isolated from caprine (goat) milk

Antibiotic Name	Potency	Goat		
		R (%)	I (%)	S (%)
Amoxicillin	10µg	44.44	11.11	44.44
Cefoxitin	30µg	0.000	11.11	88.89
Linezolid	30µg	0.000	22.22	77.78
Amoxicillin+ Clavulanic acid	20µg	22.22	11.11	66.67
Vancomycin	30µg	44.44	33.33	22.22
Oxytetracycline	30µg	0.000	0.000	100
Chloramphenicol	10µg	11.11	22.22	66.67
Trimethoprim-Sulphmethoxazole	25µg	0.000	11.11	88.89
Gentamicin	10µg	0.000	0.000	100

Note: R= Resistant, I= Intermediate, S= Sensitive

3.4 *In-vitro* Therapeutics Efficacy of Various Antibiotics against Methicillin Sensitive *Staphylococcus aureus* Isolated from Pets

The *In-vitro* findings of current study revealed Oxytetracycline, Amikacin, and Gentamicin presenting 100%, Chloramphenicol, Ciprofloxacin, and Trimethoprim-Sulphmethoxazole presented >80% efficacies against MSSA isolated from cat and dog. However, vancomycin and ampicillin presented 70% and 90% resistance against MSSA obtained from cat while 85.71% resistance was noted from both antibiotics against MSSA of dog origin. Fusidic acid showed 30% and 57.14% resistance against MSSA isolated from cat and dog respectively. Varying degree of sensitivity of antibiotics against MSSA isolated from pets (cat, dog) was observed during the study as mentioned in (Table 4)

Table 4. *In-vitro* therapeutics efficacy of various antibiotics against methicillin sensitive *Staphylococcus aureus* isolated from pets

Antibiotic Name	Potency	Cat			Dog		
		R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Vancomycin	30ug	70.00	20.00	10.00	85.71	0.000	14.29
Ampicillin	10ug	90.00	10.00	0.000	85.71	14.29	0.000
Chloramphenicol	30ug	0.000	20.00	80.00	0.000	0.000	100
Fusidic acid	10ug	30.00	50.00	20.00	57.14	42.86	0.000
Ciprofloxacin	5ug	0.000	20.00	80.00	0.000	14.29	85.71
Oxytetracycline	30ug	0.000	0.000	100	0.000	0.000	100
Trimethoprim-Sulphmethoxazole	25ug	10.00	0.000	90.00	14.29	0.000	85.71
Amikacin	30ug	0.000	0.000	100	0.000	0.000	100
Gentamicin	30ug	0.000	0.000	100	0.000	0.000	100

Note: R= Resistant, I= Intermediate, S= Sensitive

4. Discussion

The present study found 26.66% and 31.66% prevalence of MSSA from buffalo and cattle milk respectively. A study conducted by [31] on methicillin resistant and susceptible staphylococci from bovine milk in China found 52.80% (113/214) prevalence of MSSA that is higher than the findings of current study. The present study found 65.33% and 69.33% prevalence of MSSA from dogs and cats respectively. A study conducted by [32] on prevalence of MRSA and MSSA among the staff and pets in a small animal referral hospital UK. [32] found 6.66% and 33.33% prevalence of coagulase positive MSSA from dogs and cats respectively. Another study conducted by [33] found 7.85% (46/586) prevalence of MSSA from pets that is lower than the findings of current study. Another study conducted by [34] found 70% of *S. aureus* cat isolates were sensitive to methicillin (MSSA) that is similar with the findings of current study. The higher prevalence of MSSA in this area could have been because of less use of beta-lactam antibiotics, geographical variation, influence of genetic and environmental factors [35] In current study, methicillin susceptible *S. aureus* was found 83.3% which is in order with the previous results 90.8% as discussed by [36], 80% published by [37], 84% by [38]herd prevalence of *S. aureus*, including MRSA, was estimated from bulk tank milk (BTM and 98% described by [39]. Another study conducted by [40] on MRSA and MSSA from caprine (sheep) milk found 53.5% prevalence of MSSA that is lower than the findings of current study (83.33%).

The findings of present study revealed Oxytetracycline and Gentamicin presented 100%, and Ciprofloxacin

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showed 50% efficacies against MSSA isolated from both cattle and buffalo milk. These results are in line with the previous research showing more than 85% sensitivity of MSSA isolates against tetracyclines reported by [41]and 100% susceptibility to oxytetracycline by [42]. The excellent response to gentamicin observed during this study is supported by [43] which may be linked with limited use of gentamicin in late 1990's and apparent shift in MSSA isolates. However, Trimethoprim-Sulphmethoxazole and Vancomycin showed 30% and 23.08% efficacy against MSSA obtained from buffalo while no efficacy was noted against MSSA of cattle origin. These results are comparable to results reported by [44]which encodes a two-component signaling pathway whose activating ligand is an agr-encoded autoinducing peptide (AIP) in which higher percentage of intermediate or sensitive strains to trimethoprim + sulfamethoxazole was noted. Some studies reported very lower percentages of resistant isolates as conducted by [45]isolated from 54 samples of raw milk and dairy products of bovine, ovine, caprine and bubaline origin were tested for the presence of genes coding for staphylococcal enterotoxins (SEs/SEls which found 1.3% of resistant isolates. Vancomycin resistance shown in the MSSA isolates is in line with previous studies because it is an emerging issue in MSSA isolates which may be due to the acquired resistance just like methicillin [46]. The present study found 100% resistance and intermediate variants of fusidic acid and enoxacin against MSSA of buffalo and cattle origin respectively. Amikacin efficacy was increased from 33.33% to 100% against MSSA isolated from buffalo milk as compared to cattle milk. High resistance to Fusidic acid in MSSA isolates is similar to results reported by [47]. Remarkable resistive response of MSSA to Fusidic acid is due to mutations in *fus* gene islands resulting in amino acid substitutions of protein encoded [48]. Deceasing multidrug resistance in community clinical isolates especially in MRSA is due to successful identification and treatment protocol, frequent multidrug therapy, specificity for control, contact precautions, active surveillance and adjunctive control measures adoption [7,49].

The current study *In-vitro* antibiotic trial against MSSA isolated form cats presented 90%, 30%, 0.0%, 0.0% and 10% resistance by ampicillin, fusidic acid, ciprofloxacin, oxytetracycline and trimethoprim-sulphamethoxazole respectively with almost similar resistance pattern was noted against MSSA of dog origin. The similar type of findings were also reported by [32]. Our study indicated that MSSA isolates were 100% sensitive to Gentamicin and Oxytetracycline, and 88.89% to Trimethoprim + Sulphamethoxazole and Cefoxitin. These results were similar to results reported by [50,51] who reported 80-100% sensitivity of *S. aureus* against these antibiotics except cefoxitin sensitivity which is in line with the results reported by [52-54]. The general trend of sensitivity shown by Chloramphenicol, Amoxicillin+Clavulanic acid, and Amoxicillin was in the range of 40-70% in current study. These results are comparable to [7,52]. In our study, MSSA isolates exhibited 77.78% sensitivity to Linezolid, which is comparable to results reported by [7]. However, MSSA showed lesser sensitivity to Vancomycin 22.22%, which may be developed due to use of Vancomycin as last choice in the treatment of *S. aureus* infections [52]. The development of antibiotic resistance in *S. aureus* strains is an alarming situation in dairy goats. Although the antibiotic sensitivity results described here are comparable to earlier studies in which *S. aureus* were mostly sensitive to Vancomycin, Chloramphenicol, and Cefoxitin [55]. Because these drugs are not commonly used in veterinary medicine to treat *S. aureus* infections in goats [56].

5. Conclusion

The present study found overall higher prevalence of MSSA isolated from caprine (83.33%), and pets (cat 69.33%; dog 65.33) while lower prevalence from bovine origin (buffalo 26.66%; cattle 31.66%). The higher percentage of MSSA was found from caprine as compared to bovine and pets. *In-vitro* antibiotic therapeutic efficacy indicated amikacin, oxytetracycline, and gentamicin presented higher sensitivity to MSSA isolates from all origins while vancomycin and ampicillin exhibited higher resistance against MSSA isolates from all sources with fusidic acid, amoxicillin and ampicillin resistance against MSSA isolates from bovine, caprine and pets respectively. The study found variable response to antibiotics in addition to higher prevalence of MSSA from bovine, caprine and pet interface.

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ARTICLE

Dietary Cucumis melo Reduces Markers of Muscle and Articular Inflammation Following High-intensity Exercise in Horses

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ABSTRACT

We evaluated the antioxidative and anti-inflammatory potential of daily oral supplementation with a proprietary powdered *Cucumis melo* pulp (CMP) on exercise-induced markers of articular and muscular oxidative stress and inflammation in 12 horses. Horses performed a high-intensity exercise test immediately prior to, and then following, 3 weeks of daily supplementation of 1 g powdered CMP (CMP; n=8). Controls (Co; n=8) underwent the same exercise and sampling regime but were not supplemented. Blood and synovial fluid (SF) samples were taken 24 h prior to exercise (BL), and at 1 and 24 h following exercise. Plasma and SF were analysed for prostaglandin E_2 (PGE_2), total antioxidant status (TAS), nitrite and superoxide dismutase (SOD) activity. SF was analysed for glycosaminoglycans (GAG), and plasma was analysed for thiobarbituric acid reactive substances (TBARS). Comparisons were made using repeated measures with the initial exercise test as a covariate. There was an increase in SF SOD activity in the CMP group. Compared to Co at 1 h, CMP reduced nitrite and GAG in SF, as well as maintained plasma TAS and lymphocyte levels. At 24 h, plasma PGE_2 and creatine kinase were lower in horses receiving CMP. Three weeks of supplementation with CMP reduced markers of articular and skeletal muscle oxidative stress and inflammation in response to high-intensity exercise in horses. Nutritive antioxidants may provide a useful adjunct to the daily nutrition plan of horses undergoing regular exercise training and competition.

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Abbreviations

A:G ratio	albumin:globulin ratio
ALB	albumin
AST	aspartate aminotransferase
BL	baseline
CK	creatine kinase
CMP	*Cucumis melo* pulp powder
Co	control
Cre	creatinine
GAG	glycosaminoglycans
HGB	hemoglobin
IU	International Units
LYM	lymphocytes
NRC	National Research Council
PGE_2	prostaglandin E_2
RBC	red blood cells
ROS	reactive oxygen species
SF	synovial fluid
SOD	superoxide dismutase
TAS	total antioxidant status
TBARS	thiobarbituric acid reactive substances.

1. Introduction

Horses that have performed moderate to high intensity exercise experience increased mitochondrial production of reactive oxygen species (ROS; oxygen free radicals) within muscle [1,2]. An accumulation of ROS appears to be a key contributor to the inflammatory response to intense exercise that is evident both in muscle [2] and in blood [1-7]. In horses, the magnitude and duration of oxidative stress markers in plasma and synovial fluid was proportional to the exercise intensity and duration [3,7,8]. One of the first studies to relate performance to inflammation was that of Davis et al. [9] using humans, in a trial where curcumin was provided as a nutritive antioxidant. Similar results were recently demonstrated when people consumed quercetin for 14 days [10]. Improved performance has also been shown to be a sequalae of reduced muscle damage [11], inferring that inflammation is associated with decreased performance. Yang et al. [12] showed within-game deterioration of performance has also been directly associated with increased muscle inflammation. Tanabe et al. [13] demonstrated that dietary curcumin attenuates acute inflammation and muscle damage and could facilitate faster recovery. Taken together, these results demonstrated that the inflammation associated with the oxidative stress of exercise can impair performance and rate of recovery from normal exercise training bouts [14,15].

Within the joints, the stress of exercise training or conditioning is also often associated with increased ROS within synovial fluid and inflammation around and within the joints [16]. This is evidenced by elevated levels of synovial fluid markers of oxidative stress and inflammation such as prostaglandin E_2 (PGE_2) [17,18]. Excess production of ROS (such as superoxide and hydrogen peroxide) within joints directly contributes to the inflammation [17,19,20]. While a mild inflammation may be deemed a normal part of the beneficial adaptive responses to exercise, excessive inflammation contributes to muscle and joint pain, further tissue degeneration, and impaired exercise performance and wellness [21,22].

The incidence of degenerative joint disease, eventually leading to clinical osteoarthritis (OA), is high in young performance horses, often at a time when these athletes should be at the peak of their careers [2,8]. Increasingly, veterinarians, owners and trainers are using dietary or nutritive antioxidant supplements to mitigate inflammation associated with excess oxidative damage [23]. The dried and powdered pulp of a particular non-GMO strain[a] of *Cucumis melo* LC (cantaloupe or muskmelon) is rich in the antioxidant enzymes superoxide dismutase (SOD) and catalase [24-26] and has been shown to provide protection against administered pro-inflammatory compounds [25]. This *C. melo* pulp (CMP) also reduced markers of oxidative stress and improved antioxidant activity in humans [27], pigs [28], felines [29], mice [30] and horses [31]. In a recent study CMP was one of the ingredients in an oral supplement for horses that performed the same study as described herein [23]. They also reported reductions in the markers of muscle and synovial fluid oxidative stress and inflammation.

The objective of the current study was to investigate if CMP, one of the ingredients used in the previous study [23] was associated with antioxidant and anti-inflammatory effects when fed daily to horses for 3 weeks. It was hypothesized that CMP will result in reduced signs of muscle and/or articular markers oxidative stress and inflammation following high-intensity exercise.

2. Methods

2.1 Ethical Approval

All experimental procedures and protocols were approved

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by the Nutraceutical Alliance Animal Care Committee (Campbellville, ON, Canada) prior to the beginning of this study in accordance with the Ontario Animals for Research Act and the Canadian Council on Animal Care guidelines. All horses were privately owned, and written informed consent was obtained from each owner prior to the start of the study.

2.2 Experimental Animals

Twelve horses of mixed breed, gender (6 mares, 6 geldings), and age (range 5 - 17, median 7.5) participated in this study (Table 1). Stratified assignment of horses to groups was performed so that groups were as uniform as possible according to body mass, body condition score, age, sex, breed and age. All horses were clinically normal and underwent weekly health checks which consisted of checking rectal temperature, heart rate, respiratory rate, hydration (by skin pinch), menace reflex, and gut sounds. Health checks were also performed on all horses within 24 h prior to each sampling day. All horses had no known veterinary history of chronic joint inflammation or lameness and were visibly sound on exercise days using the American Association of Equine Practitioners lameness exam scoring system. Prior to participation in the study, and during the study, all horses maintained a regular schedule of pleasure riding and light lessons. Horses were randomly assigned to a diet containing either 0 (Co; n=8) or 1 g (CMP; n=8) in their morning feed, based on the manufacturer's instructions. The supplement dosage was therefore in the range of 1.55 to 2.14 mg / kg body mass per day. The supplement provided approximately 2600 IU SOD per horse/day according to the manufacturer. All horses were fed a diet which met their nutritional requirements [32] and were housed at the same facility in a loose-housing system, with unlimited access to shelter, pasture, hay and water.

Table 1. Description of horse breed, age, gender, treatment, and round for control (Co, n=8) and supplemented (CMP, n=8) horses used in the current study

Horse	Breed	Gender	Age, yr	initial, final mass (kg)	Group	Round
1	Thoroughbred	Gelding	8	467, 477	Co	1
2	Quarter horse	Mare	5	565, 566	Co	1
3	Dutch warm-blood	Mare	5	585, 583	Co	1
4	Thoroughbred	Mare	13	571, 582	Co	2
5	Morgan	Gelding	16	504, 516	CMP	1
6	Oldenburg	Mare	7	591, 584	CMP	2
7	Quarter horse	Gelding	7	579, 585	CMP	3
8	Thoroughbred	Gelding	13	473, 466	CMP	3
9	Morgan	Gelding	17	506, 496	Co, CMP	2,1
10	Quarter horse / Haflinger	Mare	5	501, 508	Co, CMP	2,3
11	Thoroughbred	Gelding	7	541, 542	Co, CMP	2,1
12	Quarter horse	Mare	8	645, 647	Co, CMP	3,2

2.3 Test Material

The test material was a 1 kg sample of a fresh batch of CMP provided by Lallemand Animal Nutrition in a sealed vacuum pouch (registered as Melofeed® in Europe and as Enzalox™ in North America; Lallemand Animal Health, Montreal, Canada). The pouch was opened on the first day it was to be used as a supplement to the feed, and thereafter maintained in a sealed plastic container in a refrigerator (6 – 10°C). The dry, powdered product is stable for at least 2 years when maintained under these conditions. This sample provided adequate supplement for the entire study.

2.4 Study Design

This study was part of a larger 3-way crossover design [23] that was conducted over 3 rounds (Fig. 1); horses partic-

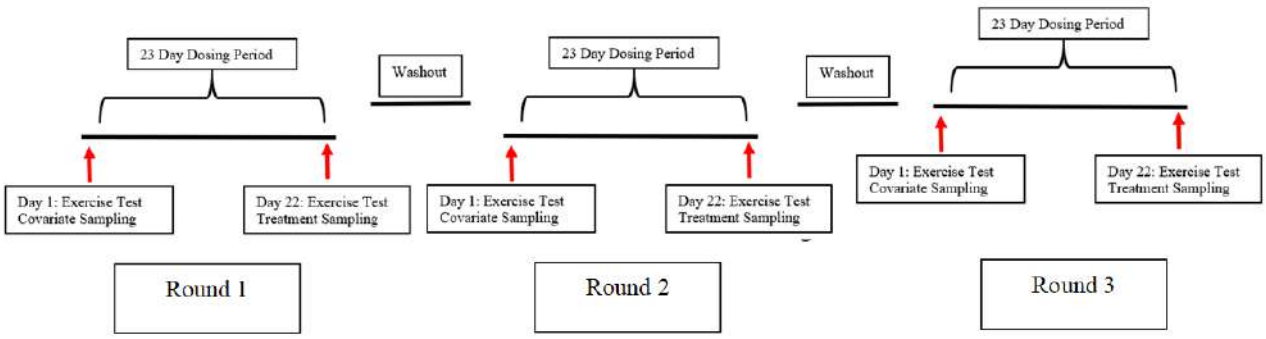

Figure 1. Schematic representation of the experimental schedule

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ipated in a control round, a CMP round, and a round for another nutraceutical treatment with a 25-day duration for each round. Only the person feeding the horses knew if which horses received supplement, and horse owners were also blinded to the treatment, by consent. Each round consisted of a covariate sampling period, a dosing period, and a treatment sampling period. For each round, a high-intensity exercise test was performed on day 1 (covariate sampling period), followed by 23 days of supplementation (or placebo) provided in the morning meal (dosing period), and a second exercise test on day 22 (treatment sampling period). The purpose of the day 1 exercise test was to serve as a time-based control, as well as provide a baseline. This was incorporated into the statistical analysis as a covariate. Horses maintained their usual activity and training level for the entire duration of their participation in the study. Rounds were separated by a 3-wk washout period to minimize nutritional, biochemical and physiological carryover effects [33-35]. The order of the treatments was randomized across the cohort of horses (Table 1).

By design, it was intended that each horse was to complete each of three treatments trials: control, CMP and a blended product of which the results have already been reported [23]. However, as in most moderate-duration field-type studies, and particularly those with a high intensity exercise component with repeated arthrocentesis, there was some attrition of horses that started. It is important, physiologically and psychologically, that horses are calm and easily manageable for all procedures, else anxiety will be a major confounder of physiological variables. Thus, the first two rounds consisted of 6 horses per round (3 Co, 3 CMP), and the last round consisted of 4 horses (2 Co, 2 CMP), due to subject attrition unrelated to the study (see Table 1). Of the 12 horses, 4 participated in the control group (Co), 4 participated in the CMP group (CMP), and 4 participated in both (Co and CMP).

2.5 Exercise Test

Horses were exercised under saddle in groups of 2 or 3 matched according to speed and fitness at approximately 11 am. Three riders were used, of similar body mass (+/- 10 kg) and riding experience (competent, non-professional). Ambient conditions were dry, with relative humidity 50 and 70% and temperature 14 – 22^{o} C. Heart rate was monitored during galloping by the riders using equine heart rate monitors (Polar Electro Canada, Lachine, QC, Canada). Horses were warmed up (at the walk, trot, and canter) for 20 min prior to the exercise test. The exercise test consisted of repeated single-lap gallops around a dry ½ mile dirt track at a target heart rate of 180 bpm, separated by approximately 4 min of walking until heart rates decreased to approximately 100 bpm [36]. This process was repeated for 2-3 more laps (depending on the ability of the horse) until the horse's time around the track increased by 10% of their fastest lap. The first exercise test sequence performed by each horse was used to determine their maximal heart rate, speed, and ability. The mixed breed horses were able to complete 3 laps while the Thoroughbreds completed four. The number of laps was conserved for each horse for the subsequent exercise tests.

2.6 Sample Collection

Samples were obtained at baseline (BL), around 11 am the day prior to each of the exercise tests, then again at 1 and 24 h following the cessation of exercise. Timing of sampling was determined in a previous study that examined the time course of inflammation and oxidative stress markers in response to high intensity exercise in horses [36].

An area approximately 4 X 5 cm was clipped around the blood sampling site (left jugular groove), and synovial fluid sampling sites (the medial side of the left and right intercarpal joints) to reduce hair length to less than 2 mm. Topical anesthetic (Emla cream, 2.5% lidocaine, 2.5% prilocaine; AstraZeneca, Mississauga, ON, Canada) was applied to the clipped area approximately 30 min prior to sampling. Blood samples were collected directly into sodium heparin and EDTA-vacutainer tubes (Becton-Dickson, Mississauga, ON, Canada) from the jugular vein using a 21G 1.5" multiple sample needle (Becton-Dickson, Mississauga, ON, Canada). Blood samples were taken immediately prior to arthrocentesis and chilled on ice until processing, which occurred within a 2 h period.

Aseptic arthrocentesis was performed at each time point, alternating joints between samples to allow sufficient time for replenishment of SF. The left or right intercarpal joint was prepared using a stanhexidine / iodine scrub followed by 99% isopropyl alcohol. Approximately 1 mL of fluid was aspirated using a 22 G x 1" needle into a 3 cc sterile syringe. Aspirated fluid was immediately transferred into a sodium heparin vacutainer tube and chilled on ice until processing, which occurred within 2 h.

2.7 Sample Processing

Fresh blood samples were analyzed for biochemistry [albumin (ALB), albumin/globulin ratio (A:G), aspartate amino transferase (AST), creatinine (Cre), creatine kinase (CK)] (Johnson & Johnson, Ortho Clinical Diagnostics Model 5.1 Fusion) and complete blood count (Siemens Model Advia 2120, Nassagaweya Veterinary Laboratory Services, Campbellville, ON, Canada). Additional aliquots of heparinized blood and SF were centrifuged at 6000 x g

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for 15 min. Supernatant was then transferred into Eppendorf tubes and stored at -20ºC until analysis.

Prior to analysis, SF samples were digested via hyaluronidase treatment to improve assay precision [37]. Hyaluronidase (Sigma-Aldrich, Oakville, ON) was suspended in PBS to a final concentration of 4 mg/ml solution. SF samples were prepared using a 1:1 dilution of hyaluronidase solution and sample. Mixed hyaluronidase:SF samples were agitated for 1 h, centrifuged at 1000 x g for 5 min, and the supernatant was removed and used in assays.

2.8 Sample Analyses

Samples were analysed for nitrite (Griess Reaction, Molecular Probes, Eugene OR), total antioxidant status (TAS; Cayman Chemical, Ann Arbor, MI), superoxide dismutase (SOD; Trevigen, Gaithersburg, MD), and prostaglandin E_2 (PGE_2; DetectX Prostaglandin E2 Enzyme Immunoassay, Arbor Assays, Ann Arbor, MI). Plasma was analysed for thiobarbituric acid reactive substances (TBARS; R&D Systems Inc., Minneapolis, MN), and untreated SF was analysed for glycosaminoglycans (GAG) [38] as detailed previously [20].

Table 2. Time, treatment, and treatment by time effects within plasma markers of oxidative stress and inflammation, as well as blood biochemistry and hematology parameters in response to high-intensity exercise in control (Co; n=8) and supplemented (CMP; n=8) horses

	BL	1 h	24 h	p_{time}	p_{trt}	$p_{trt*time}$
SF						
SOD IU/ml						
Co	166 ± 13.0	170 ± 13.0	187 ± 13.0	0.4	0.04	0.7
CMP	183 ± 13.0	204 ± 13.0	198 ± 13.2			
PGE_2 pg/ml						
Co	196 ± 31.6	227 ± 32.1	163 ± 31.6	0.04	0.3	0.2
CMP	171 ± 30.8	144 ± 30.5	128 ± 30.5^{a}			
Plasma						
TAS mM						
Co	1.09 ± 0.023^{a}	0.97 ± 0.023^{b*}	1.06 ± 0.024^{ab}	0.004	0.1	0.1
CMP	1.07 ± 0.022	1.04 ± 0.024	1.09 ± 0.023			
Blood						
A:G						
Co	0.92 ± 0.035	1.04 ± 0.053	0.91 ± 0.044	0.004	0.6	0.8
CMP	0.96 ± 0.033	1.04 ± 0.051	0.93 ± 0.043			
ALB, g/dL						
Co	31.4 ± 0.54	34.8 ± 1.65	31.9 ± 0.64	0.0062	0.6	0.3
CMP	32.2 ± 0.53^{ab}	36.3 ± 1.62^{a}	31.6 ± 0.65^{b}			
AST, µmol/L						
Co	342 ± 25.8	399 ± 34.2	490 ± 74.2	0.013	0.7	0.4
CMP	401 ± 26.1	417 ± 33.2	468 ± 71.0			
CRE, U/L						
Co	117 ± 5.0^{a}	137 ± 5.5^{b}	119 ± 5.0^{a}	<0.0001	0.9	0.7
CMP	114 ± 4.8^{a}	139 ± 5.0^{b}	121 ± 5.0^{a}			
HGB, g/L						
Co	13.7 ± 0.55^{a}	16.0 ± 0.59^{b}	15.3 ± 0.55^{b}	0.0005	0.7	0.4
CMP	14.0 ± 0.54^{a}	15.9 ± 0.55^{b}	14.5 ± 0.55^{ab}			
RBC, 10^{12}/L						
Co	8.1 ± 0.29^{a}	9.6 ± 0.33^{b}	9.0 ± 0.29^{ab}	0.0004	0.4	0.6
CMP	8.1 ± 0.30^{a}	9.3 ± 0.29^{b}	8.5 ± 0.30^{ab}			
LYM, 10^{9}/L						
Co	2.9 ± 0.30^{a}	4.8 ± 0.30^{a}	3.0 ± 0.30^{a}	≤ 0.006	0.002	0.01
CMP	3.0 ± 0.28	3.3 ± 0.30*	3.3 ± 0.30			

Marker concentrations are means ± SEM. BL = baseline sample taken approximately 24 h prior to the exercise test; SOD = superoxide dismutase; TAS = total antioxidant status; A:G = albumin to globulin ratio; ALB = albumin; AST = aspartate aminotransferase; CRE = creatinine; HGB = hemoglobin; RBC = red blood cells; LYM = lymphocytes.

Notes: [ab] Superscripts which differ denote significantly different values within treatment ($p \leq 0.05$).

* Denotes a significant difference between Co and CMP at the particular timepoint ($p \leq 0.05$).

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2.9 Statistical Analysis

Data are presented as mean ± SEM unless otherwise stated. Data were analyzed with respect to pre- vs post-supplementation / placebo within each group, exercise time points, and trial using analysis of variance (ANOVA). Marker concentrations (Yijk) were subject to repeated measures ANOVA (PROC MIXED University Edition, SAS Institute Inc.) according to the following model:

$$Yijk = \mu + \beta \times initial + trti + timej + trt*timeij + \varepsilon ijk$$

where μ = overall mean, β = the covariate slope, initial = marker concentration during the covariate sampling period, trt = fixed effect of treatment (i = Co or CMP), time = repeated measure of time (j = 1 to 3) and εijk = the experimental error. Comparisons were also made between treatments at each sampling time using estimate statements. Least square means using a Tukey adjustment were employed to analyse the effects of time within group. Significance was set at $P \leq 0.05$. In the description of the results the term 'time effect' therefore indicates that the 3 weeks of supplement resulted in an effect that can be, and has been, attributed to the supplement. The term 'treatment effect' refers to differences between the control group and the treatment group at the second time point (end of trial).

3. Results

3.1 SF Markers of Oxidative Stress

No effects were observed for SF TAS (Table 2). There was an effect of trial duration on SOD activity, which was lower at the end of the trial period before exercise in the control trial but not the CMP trial (data not shown). There was an effect of treatment for SF SOD, with consistently higher SF SOD in the CMP group than the Co group (p = 0.04, Table 2).

3.2 SF Markers of inflammation

There were no effects of trial duration on SF markers of inflammation (pre-exercise comparisons). SF PGE_2 was significantly lower at 24 h post exercise in CMP resulting in an overall time effect (p = 0.04, Table 2). There were no significant differences between treatments at 1 h post-exercise (227 ± 32.1 pg/ml in Co vs 144 ± 30.5 pg/ml in CMP; p = 0.09). There was an effect of time for SF fluid nitrite (p = 0.04). Nitrite was lower in CMP (9 ± 7.7 μM) compared to Co (39 ± 7.7 μM) at 1 h (p = 0.01, Figure 2). Synovial fluid GAG was lower in CMP (344 ± 155 μg/ml) compared to Co (820 ± 130 μg/ml) at 1 h (p = 0.03, Figure 3).

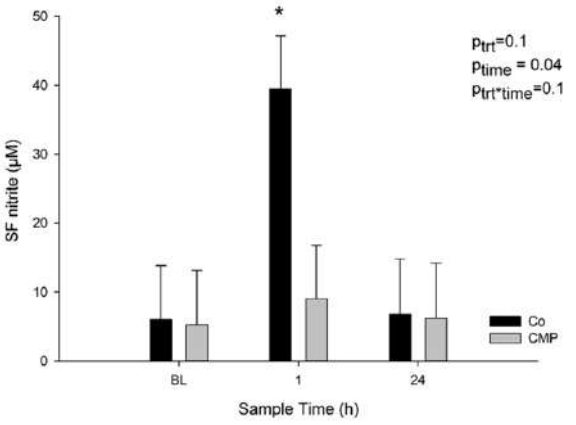

Figure 2. Mean ± SEM of synovial fluid (SF) nitrite (μM) in control (Co; n=8) and supplemented (CMP; n=8) horses 24 h prior to (BL), at 1 and 24 h following, a standardized high-intensity exercise test

Note: * Denotes significant difference between the Co and CMP (p≤0.05).

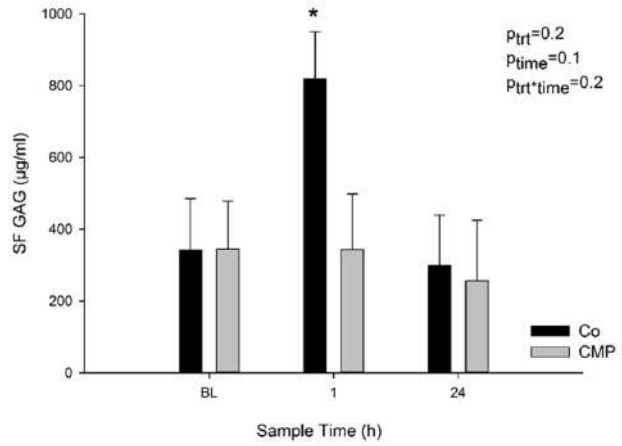

Figure 3. Mean ± SEM of synovial fluid (SF) glycosaminoglycans (GAG; μg/ml) in control (Co; n=8) and supplemented (CMP; n=8) horses 24 h prior to (BL), at 1 and 24 h following, a standardized high-intensity exercise test

Note: * Denotes significant difference between the Co and CMP (p≤0.05).

3.3 Plasma Markers of Oxidative Stress

There were no effects of trial duration on plasma markers of oxidative stress (pre-exercise comparisons). There was an effect of time on plasma TAS (p = 0.004), and in the Co plasma TAS was lower compared to BL at 1 h (p = 0.01) and compared to CMP at 1 h (p=0.03, Table 2).

3.4 Plasma Markers of Inflammation

There were no effects of trial duration on plasma markers of inflammation (pre-exercise comparisons). Plasma PGE_2 was reduced in CMP (128 ± 30.5 pg/ml) compared to Co (163 ± 31.6 pg/ml) at 24 h (p = 0.04, Figure 4).

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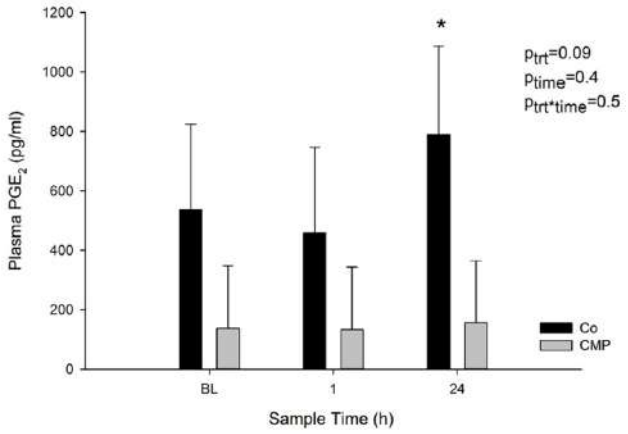

Figure 4. Mean ± SEM of plasma prostaglandin E2 (PGE_2; pg/ml) in control (Co; n=8) and supplemented (CMP; n=8) horses 24 h prior to (BL), at 1 and 24 h following, a standardized high-intensity exercise test

Note: * Denotes significant difference between the Co and CMP ($p \leq 0.05$).

3.5 Markers of Blood Biochemistry

Time effects were observed for CRE, AST, ALB, A:G ratio, HGB, and RBC (Table 2). There was an effect of treatment ($p = 0.02$) and time ($p = 0.02$) for CK. In the Co CK was higher compared to BL at 24 h (665 ± 73.0 U/L vs 295 ± 76.8 U/L; $p = 0.03$) and CMP at 24 h (353 ± 77.6 U/L; $p = 0.008$, Figure 5).

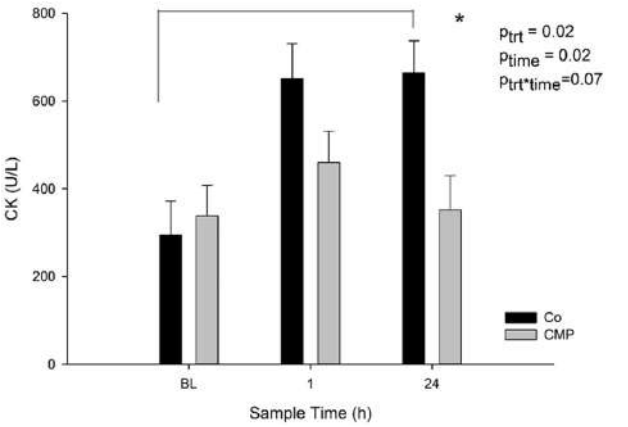

Figure 5. Mean ± SEM of creatine kinase (CK; U/L) in control (Co; n=8) and supplemented (CMP; n=8) horses 24 h prior to (BL), at 1 and 24 h following, a standardized high-intensity exercise test

Notes: Lines connect significantly different time points within the Co.
* Denotes significant difference between the Co and CMP ($p \leq 0.05$).

3.6 Markers of Immunity

Time ($p = 0.004$) and time by treatment ($p = 0.01$) effects were observed for blood lymphocytes (Table 2, LYM). In the Co, LYM was elevated at 1post-exercise, and this response was not observed in the CMP trial. In the CMP trial, there was no effect of exercise on the LYM response. There were no changes in any of the other hematological variables.

4. Discussion

This study provides evidence that dietary CMP behaved as a nutritive antioxidant and influenced the systemic and articular environment in horses undergoing and recovering from intense exercise. Given that muscle mass is about 40% of body mass, and the largest single tissue in the body [39] and that the horses exercised at high intensity using muscle, the systemic responses observed in the present study can only be attributed to skeletal muscle. Horses supplemented with CMP displayed attenuation of post-exercise increases in SF nitrite and GAG, as well as evidence of diminished plasma PGE_2 and CK. These results are consistent with reductions of oxidative stress and inflammation in joints and skeletal muscle after high intensity exercise. The results also confirm that antioxidant and anti-inflammatory responses observed in the equine joint oral supplement reported previously [23] may be attributed in part to CMP.

There was a general absence of effect of CMP supplementation on measures obtained at rest (pre-exercise samples on day 1 compared to pre-exercise on day 22). This suggests that in the absence of a measurable oxidative stress a physiological or biochemical adaptation to 3 weeks of supplementation was not readily apparent. The observed time effects in blood variables are consistent with physiological changes due to high-intensity exercise [7,36,40] indicating that the standardized exercise test was of suitable intensity and physiological stress. Additionally, in Co horses, plasma TAS was reduced 1 h following the exercise test indicating that the exercise was of sufficient intensity to tax and diminish antioxidant systems. Short intense bursts of physical effort in horses [3,5,40], endurance exercise in dogs [41] and horses [8,42], acute bouts of treadmill exercise in rats [43] and high-intensity leg cycling exercise in healthy adult humans [44] have also resulted in decreased plasma TAS.

In contrast, following 23 days of CMP the maintenance of plasma TAS at 1 h post-exercise suggests a reduction of oxidative damage, inflammation, and muscle membrane injury (evidenced by reduced plasma PGE_2 and CK 24 h following exercise). A nutritive antioxidant that mitigates excessive oxidative stress and inflammation [2,6,20,45] is appealing for endurance horses and horses that are required to compete for multiple days. However, care must be taken to also allow normal muscle repair and growth mechanisms to occur [46].

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Lymphocyte concentrations and plasma TAS were maintained at baseline levels in CMP supplemented horses 1 h post-exercise, in contrast to the elevation seen at 1 h post-exercise in Co. Within the Co group, TAS was significantly decreased at the 1 h time point compared to BL. While there were no overt effects of CMP supplementation in horses at rest (pre-exercise comparison 22 days apart) CMP supplementation appears to have assisted in maintaining blood antioxidant potential and this became evident in response to the oxidative stress imposed by high intensity exercise. The mechanism is unclear as plasma SOD activity was unchanged in the CMP group, but perhaps indicating that activity was adequate for the demands. It is also possible that upregulated activities of glutathione peroxidase or catalase may have contributed to the increase in plasma TAS [25,47], and this needs to be investigated in future studies.

Ingestion of encapsulated forms of CMP reportedly mitigates the severity of diabetic neuropathy [26] and biomarkers associated with exercise stress [48]. The CMP is made from a melon with naturally very high SOD and catalase activities [26,49]. After ingestion in vegetal oil encapsulated form, as in the present study, the CMP exhibits anti-oxidant and anti-inflammatory properties *in vitro* and *in vivo* [24-26]. However the *in vivo* effects cannot be attributed to an absorption of SOD, catalase or other large protein molecules because these cannot be absorbed by the g.i. tract. Rather, dietary proteins and peptides undergo chemical modification and degradation to smaller peptides and amino acids when unprotected from the gastric environment (low pH, proteases, etc.), so it is not surprising that ingestion of unprotected CMP resulted in a loss of antioxidant parameters *in vivo* [19,24] consistent with SOD pharmacokinetics [50]. While the encapsulation (protection) of CMP protects proteins within the product from acid degradation in the stomach, the large protein molecules then present within the intestinal environment cannot be absorbed by transport systems of the intestinal epithelial cells. Based on this well known physiology, it can be concluded that the high SOD and catalase concentrations and activities of CMP cannot be directly responsible for the increased antioxidant potential in plasma and synovial fluid seen in the present study. This raises the question of how dietary CMP, when it passes through the stomach and enters the intestinal system largely intact, has the ability to induce systemic and local upregulation of antioxidant parameters. This is a topic of investigation by Lallemand (personal communication, Dr. Yannig Letreut, January 2017). What appears to be the case is that an indirect, and as yet unknown mechanism, induces the reported increases in endogenous antioxidant defense systems [24,29,49].

Excessive oxidative stress (levels of ROS that cannot be controlled by endogenous activities of oxygen free radical scavengers such as SOD, catalase and glutathione peroxidase) in skeletal muscle is associated with elevations in cytosolic calcium and membrane lipid peroxidation [46,51]. Membrane lipid peroxidation results in leakage of proteins into blood with the time course and magnitude proportional to the amount of muscle damage [52]. The exercise stress in the present and related studies [19,29] resulted in evidence of muscle damage as indicated by increases in plasma CK and AST in horses that did not receive CMP. Twenty-three days of supplementation with the blended supplement resulted in plasma CK and AST that were not significantly elevated following exercise and there was a tendency towards an upregulation in SF SOD 1 h following exercise [23]. The indications of reduced skeletal muscle damage in these studies are consistent with the reported antioxidant effects of CMP. An additive or synergistic effect of ingredients in the blended supplement may account for the more definitive antioxidant response seen previously [23].

Post-exercise muscle soreness, stiffness, reduced muscle force production and joint range of motion (DOMS – delayed onset muscle soreness) do not become apparent until approximately 24 to 48 h following exercise [52]. The mitigation of plasma PGE_2 24 h following exercise in the CMP group suggests CMP supplementation may have aided in attenuating post-exercise inflammation.

Proteoglycan fragments and increased GAG occur in SF following exercise [17] and also occur as a result of injury or applied irritants [20,53]. Increased SF GAG concentration is a marker of articular cartilage degradation. While the mechanism of exercise-induced increases in proteoglycan fragments and GAG are not fully understood, excessive mechanical loading [54] and / or inflammatory response [20] may be responsible. Thus, the blunted increase in post-exercise SF GAG noted in the CMP group compared to the Co 1 h post exercise in the current study indicates a reduction in cartilage breakdown post exercise. This was associated with evidence for an improved antioxidant potential of the synovial environment in CMP horses (increased SF SOD in the CMP; reduced SF nitrite 1 h post exercise). A more comprehensive examination of SF markers of antioxidant activity both at rest and following exercise are required to understand mechanisms by which nutritive antioxidant can mitigate marker of joint stress and inflammation.

The regulatory environment for dietary supplements differs by country or region. This CMP product has been registered and approved for use as an ingredient in animal feeds in the EU (under the name Melofeed), as a veterinary health product in Canada (under the name Enzalox)

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and as an animal supplement in the United States (National Animal Supplements Council under the name Enzalox). It is important that dietary nutraceutical supplements are both safe and effective for the animals for which they are intended. The regulatory authorities have agreed that the product is safe under the intended conditions of use for food production animals and for horses, dogs and cats. The present study demonstrates efficacy of the CMP at dosages between 1.55 and 2.14 mg / kg body mass for horses.

Duration-response and dose-response studies with the CMP product have not been performed using horses, and the amount used was recommended on the basis of studies performed on production animals (cattle, swine) and approved for use by the European Food Safety Authority (EFSA). The duration selected is typical of that required for physiological and biochemical adaptations, although the time-course of response remains to be determined. It is also possible that a higher dosage may be associated with additional physiological benefit. It would also be useful to have mode of action studies that specifically examines mechanisms by which the supplement exerts the antioxidant effects at the level of muscle and in synovial joints.

5. Summary and Conclusions

Three weeks of supplementation with CMP reduced markers of articular and skeletal muscle oxidative stress and inflammation in response to high-intensity exercise in horses. There was an increase in SF SOD activity, and reduced SF nitrite and GAG 1 h following exercise in CMP horses. Plasma TAS and LYM levels in CMP horses were maintained 1 h following exercise, while PGE_2 and CK were reduced at 24 h. These data indicate that dietary CMP improved the oxidative and inflammatory responses following intense exercise. Future studies should seek to better characterize mechanisms of action of nutritive antioxidants and their roles in the physiological response to demanding exercise.

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ARTICLE

Meta-Analysis on Efficacy of Vaccination against *Staphylococcus aureus* and *Escherichia coli*

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ABSTRACT

Mastitis is a common disease responsible for the biggest economic loss in the dairy industry. Antibiotic therapy does not provide long-term protection. And residue is a major concern in food safety. Vaccination is an alternative control method with great potential for bovine mastitis. Our study focus on evaluating vaccine efficacy regarding reducing the incidence of clinical and subclinical mastitis. Meta-analysis was used to pool data extracted from previous studies. 26 records from 13 studies were examined. A fixed effect model was constructed assigning incidence as the measurement of the outcome. Risk ratio (RR) was the parameter that measured the incidence differences between treated group and control group. Studies and records were categorised based on vaccine antigens. In vaccine against *Staphylococcus aureus*, RR was 0.76; 95% CI (0.65,0.89), while in vaccine against *Escherichia coli* RR was 0.96; 95% CI (0.86,1.08).

1. Introduction

Mastitis is inflammation in quarters of the udders and the most significant cause of economic loss in dairy industry which manifests as reduced milk yield, milk composition change, reduced quality, and compromised reproduction capacity [1]. Current mainstream control method is prepartum antibiotic therapy with very limited long-term effects in reducing somatic cell counts (SCC) and increased milk yield. Bacterial antibiotic resistance and drug residues in dairy products could be the inherent risks. Vaccination function as a control method by generating long lasting immunity against offending pathogens in dairy cow, and does not have withdrawal time, which makes it a promising alternative protocol to the established ones [2,3,4]. Vaccination could be helpful as an aid for prevention of a few bacteria.

The search for effective vaccine can trace back to last century and positive result was not reported until 1980s. A vaccine, combining heat-killed capsular-type A and B *Staphylococcus aureus* and capsular polysaccharide, was reported to increase the resistance against *Staphylococcus aureus* in dairy cows, and mitigated mastitis related yield reduction [5]. About the same time, the investigation on vaccine against *Escherichia coli* also produced some positive results. J5 antigen was proven to reduce incidents of clinical coliform mastitis [6]. However, conflicting results were also reported. Some researchers argued that

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vaccines had no effect on alleviating clinical parameters like incidence rate, SCC and commercial parameters like yield loss and culling rate [7,8,9,10]. The failure of vaccine in combating mastitis could be attributed to the multiple causative pathogens [8,11], low mammary antibody titer [12], environmental factors and ect.

A previous report proposed that the implement of vaccination had some advantages [12]. With the release of the new commercial vaccine and data from new clinical trials, it is necessary to re-evaluate the mastitis vaccine efficacy and its potential application.

2. Methods

2.1 Literature Search Strategy

The literature search was conducted in Pubmed electronic database, Web of Science, in January 2018. The Medical Subject Heading (Mesh) and keywords for disease and intervention were searched in [All Fields] using various combinations. The search terms for disease were mastitis OR "bovine mastitis" and search terms for intervention were vaccine* OR vaccination*. For example, the searching strategy in Pubmed was (((Mastitis"[Mesh]) OR "Mastitis, Bovine"[Mesh])) AND (("Vaccination"[Mesh]) OR "Vaccines"[Mesh]). To supplement computer search, a manual search for possible articles was also conducted by searching references of identified articles.

The searching result was imported into Endnote X8. Duplicates were eliminated by cross check author's name, article's title and the publication year.

2.2 Inclusion and Exclusion Criteria

Studies using controlled trials and observational method like cohort study and case-control study were included. Controlled trails must consist both randomized controlled trials (RCT) and controlled clinical trials (CCT). Other inclusion criteria are: (1) participant of the study were dry or lactating cows; (2) intervention was vaccination; (3) control groups were unvaccinated or treated with placebo; (4) outcomes must include the incidence of mastitis; antibiotic treatment as control group.

2.3 Date Extraction

Trial duration, vaccination regimens and outcome measure methods were extracted. In studies containing multiple trials, if summation was available, the data in summation were extracted and all trials were considered as one study; if summation was not available, multiple-center trial was considered as one study or the data of trials were extracted separately.

2.4 Quality Assessment

The quality of RCT was assessed for the risk of bias by the Cochrane Collaboration's tool. Cochrane Collaboration's tool contains following items: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting and other bias. The first and second items assess selection bias. The third and fourth items assess performance bias. The fifth item assesses detection bias and the sixth item assesses reporting bias.

The quality of CCT was assessed by MINORS (methodological index for non-randomized studies) [13]. MINORS is particularly suitable for the evaluating non-randomized studies. There are 12 items measured in 0~2 (0 for no related report, 1 for insufficient report, 2 for sufficient report). The top eight items were for the uncontrolled groups and the rest were for the study with control groups. The last item was excluded because it assesses the statistical analysis method, while in our study, the extracted data were re-analyzed to calculate RR.

2.5 Assessment of Risk of Bias

Depending on the type of a trial, various tools were used to assess risk of bias.

2.6 Statistic Analysis

Risk ratio was used to estimate the outcome of each trial. We used STATA 14.0 to pool results with a fixed effect model. Heterogeneity was evaluated by Chi2 and I2. If I2 is below 25%, then it indicates low heterogeneity. If I2 is above 75%, it indicates high heterogeneity. When high heterogeneity was observed, data were pooled by adopting a random model. Sensitive analysis was conducted by omitting one study each time, evaluating the influence of each study on the overall effect size (RR) and the source of heterogeneity. Bias was assessed and presented by a funnel plot. The symmetry of the funnel plot revealed the extent of bias. A study with no bias is perfectly symmetrical. We included both subjective visual assessment and the objective Egger's test. The latter was conducted via the meta command in STATA 14.0 to quantitatively assess symmetry of the funnel plot and publication bias. If the p-value in each test was below 0.1, the plot was deemed asymmetric.

A subgroup analysis was conducted to evaluate the influence of the sample size. Datum was divided into two subgroups based on the calculated result of the sample size at the beginning of the analysis. The formula was shown below:

$$n = 2\frac{\left(\mu_\alpha + \mu_\beta\right)^2 P(1-P)}{(P_1 - P_0)^2}$$

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$$P = \frac{P_0 + P_1}{2}$$

P_0 was the incidence in vaccinated group. P_1 was the incidence in control group. μ_a and μ_b were t-value of type one error and type two error when df=∞.

3. Results

The literature search in electronic databases initially identified 323 studies, 279 studies were from Pubmed, 34 studies were from Sciencedirect and 10 studies from manual search. 10 duplications were eliminated. 245 studies were excluded after we examined their titles and abstracts. Then full-article sift-selection was conducted on the remaining 68 studies to verify eligibility. 58 studies were excluded for following reasons: 25 studies measured the outcome in methods incompatible to our established criteria such as the concentration of antibodies, SCC, milk yield, pathogens in milk samples and etc. 4 studies were conducted in species other than bovine, like goats and mice. 2 studies did not utilize vaccination as intervention. 2 studies had no control groups. 1 study was case control study. 8 studies were experimental challenge trial. Data in 12 studies could not be extracted. 2 studies have other incompatible objectives (Figure 1).

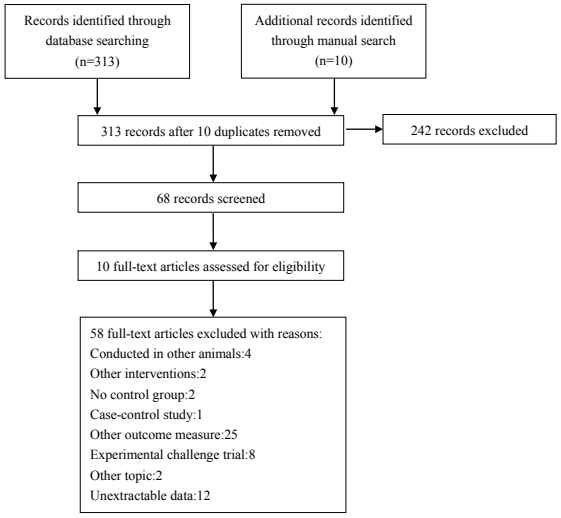

Figure 1. Flow chart search strategy

The selected studies presented the outcome with two methods, three were measured by prevalence while seven were measured by the number of cases. The duration of date recording ranged from 30DIM to 120DIM. The characteristics of the records were shown in Table 1. Different vaccination protocols were used, six of them used label regime while others used modified protocols (Table 2). The data extracted was represented in table form (Table 3a, 3b).

Table 1. Characteristics of studies included in meta-analysis

First Authors	Origin of Vaccine	Type of Control Group	Outcomes measures	Study Type
Guccione (2017,a)	Startvac	Blank Control	1.Incidence of Clinical Mastitis 2. Incidence of Subclinical Mastitis	CCT
Guccione (2017,b)	Startvac	Blank Control	1. Incidence of Clinical Mastitis 2. Incidence of Subclinical Mastitis	CCT
Bradley (2015,a)	Startvac	Blank Control	Incidence of Clinical Mastitis	RCT
Bradley (2015,b)	Startvac	Blank Control	Incidence of Clinical Mastitis	RCT
Morimoto (2011)	*E. coli*	Blank Control	Incidence of Clinical Mastitis	CCT
Wilson (2007)	*E. coli*	Blank Control	Incidence of Clinical Mastitis	CCT
Tenhagen (2001)	*S. aureus*	Placebo	Incidence of Clinical Mastitis	CCT
Hodemaker (2000)	*S. aureus*	Placebo	1.Incidence of Clinical Mastitis 2.Incidence of Subclinical Mastitis	RCT
Waston (1996,a)	*S. aureus*	Blank Control	1. Incidence of Clinical Mastitis 2.Incidence of Subclinical Mastitis	RCT
Waston (1996,b)	*S. aureus*	Blank Control	1. Incidence of Clinical Mastitis 2.Incidence of Subclinical Mastitis	RCT
Nordhaug (1994)	*S. aureus*	Placebo	Incidence of Clinical Mastitis	RCT
Mc Clure (1994)	*E. coli*	Blank Control	Incidence of Clinical Mastitis	RCT
Gonzailez (1989)	*E. coli*	Blank Control	Incidence of Clinical Mastitis	RCT

Table 2. Immunization protocols of studies included in meta-analysis

First Authors	Immunization Procotol	Immunization Route
Guccione (2017,a)	45 and 10 days before the estimated date of calving	Intramuscular Injection
Guccione (2017,b)	Label regime	Intramuscular Injection
Bradley (2015,a)	On the day of recruitment (d 0), 28 d later (d 28), 62 d thereafter (d 90), and then every 90 d until the end of the study.	Intramuscular Injection
Bradley (2015,b)	Label regime	Intramuscular Injection
Morimoto (2011)	On the day of recruitment (d 0), 30 later (d 30)	Subcutaneous Injection
Wilson (2007)	Before cows were dried off to end the previous lactation and again at 21 to 28 days before the calving due date	Subcutaneous Injection
Tenhagen (2001)	5 and 2 weeks before the estimated date of calving	Subcutaneous Injection
Hodemaker (2000)	5 and 2 weeks before the estimated date of calving	Subcutaneous Injection

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Watson (1996,a)	The last trimester of pregnancy	Intramuscular Injection
Watson (1996,b)	The end of the previous lactation	Intramuscular Injection
Nordhaug (1994)	8 and 2 weeks before the estimated date of calving	Subcutaneous Injection
Mc Clure (1994)	The first injection at drying off the second one at 2 or 3 weeks before calving	Intramuscular Injection
Gonzailez (1989)	The first injection at drying off, the second one in 28 days later the third one within 14 days after calving.	Subcutaneous Injection

Label regime: the first injection, 45 days before the predicted calving; the second injection, 35 days later (10 days before the predicted calving date); the third injection, 62 days later (52 days after the predicted calving date)

Table 3a. Date extracted from studies (vaccine against *Staphylococcus aureus*), including duration, the number of bovines in vaccinated group and control group

First Author	Duration	Vaccinated Group			Control Group		
		+	-	Total	+	-	Total
Clinical Mastitis							
Guccione(2017,a)	3M	2	28	30	2	28	30
Guccione(2017,a)	3M	0	30	30	0	30	30
Bradley(2015,a)	4M	8	550	558	8	568	576
Bradley(2015,b)	4M	10	405	415	8	568	576
Tenhagen(2000)	3M	67	97	164	74	83	157
Hodemaker(2000)	—	5	30	35	6	30	36
Watson(1996,a)	9M	41	634	675	62	663	725
Watson(1996,b)	9M	4	167	171	5	149	154
Nordhaug(1994)	—	9	49	58	10	40	50
Subclinical Mastitis							
Guccione(2017,A)	3M	4	26	30	3	27	30
Guccione(2017,B)	3M	6	24	30	12	18	30
Hodemaker(2000)	—	19	16	35	16	20	36
Total		202	2219	2421	268	2352	2620

Table 3b. Date extracted from studies (vaccine against *Escherichia coli*), including duration, the number of bovines in vaccinated group and control group

First Author	Observation Time	Vaccinated Group			Control Group		
		+	-	Total	+	-	Total
Clinical Mastitis							
Bradley (2015,a)	4M	63	495	558	57	529	576
Bradley (2015,b)	4M	48	367	415	57	529	576
Morimoto (2011)	10M	54	181	235	50	195	245
Subclinical Mastitis							
Wilson (2007)	20M	27	224	251	15	291	306
Mc Clure (1994)	5M	49	597	641	78	568	646
Gonzailez (1989)	—	6	227	233	29	198	227
Total		429	2501	2930	470	2706	3156

Note: M = month

Six RCT were included in the analysis. The result of quality evaluation was presented in Figure 2. Three out of six studies conducted random sequence generation and were evaluated as low risk [14,15,16]. Among these three studies, two utilized random number table and one utilized coin flipping. The rest did not report relevant information and therefore were deemed as unclear risk [17,7,9].

Figure 2. Qualification assessment of studies included

None of these six studies reported relevant information of whether allocation concealment was applied, therefore all of them were deemed as unclear risk.

Four studies completed blinding of participant and personnel [16,14,8,9]. Among these four studies, one coded vaccine and placebo in order to bind participant and personnel and other three studies reported clearly that participant and personnel knew nothing about allocation. The rest two studies provided no information relevant to this evaluation item and therefore were deemed as unclear risk [15,17].

Six studies completed blinding of outcome assessment. The participant and personnel of the four studies men-

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tioned above also conducted outcome assessment and therefore were evaluated as low risk. Other two studies assessed the outcomes by clinical symptoms, SCC, bacterial culture and therefore the process of the outcome assessment were considered objective.

All six studies reported complete outcome data. Information regarding exclusion of participants during the experiment and its reason was presented clearly. All six studies have no bias of selective reporting or bias of other types.

Four CCT were included. The result of quality assessment was shown in the table 4. The total scores ranged from 13 to 17. For 6th item, four out of six studies scored 1 for the lack of explicit standards of time monitoring. Only one study conducted objective evaluation of mastitis. No study estimated sample size.

Table 4. Qualification assessment of studies included using MINORS

Author	○1	○2	○3	○4	○5	○6	○7	○8	○9	○10	○11	total
Guccione (2017)	2	2	2	2	2	1	2	0	0	2	2	17
Morimoto (2011)	2	2	2	1	0	1	2	0	0	2	2	14
Wilson (2007)	2	2	2	0	0	1	2	0	0	2	2	13
Tenhagen (2001)	2	2	2	0	0	1	2	0	2	2	2	13

The pathogen in seven studies was *Staphylococcus aureus*. Twelve groups of datum were extracted from these seven studies. 4530 cows were included after pooling, 2166 of them were vaccinated, and 2364 received placebo or no treatment. One group of datum was excluded because the incidence in both vaccinated and control group was zero, and RR was not available. Low heterogeneity between was observed with I2=0% (Figure 3). The overall RR was 0.86(0.72, 1.02).

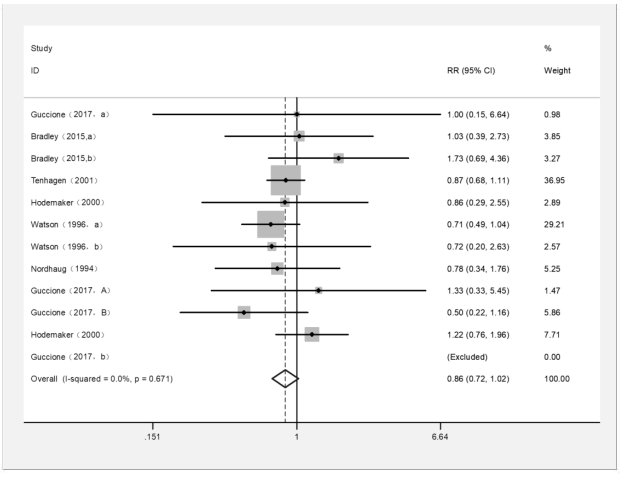

Figure 3. A forest plot of risk ratios and 95% confidence intervals for 12 records assessing the efficacy of *Staphylococcus aureus* vaccines

The pathogen in five studies was *Escherichia coli*. Six groups of datum were extracted from these seven studies. 4914 cows were included after pooling, 2338 of them were vaccinated, and 2576 cows received placebo or no treatment. High heterogeneity between was observed with I2=82.8% (Figure 4). The overall RR was 0.96 (0.81, 1.12).

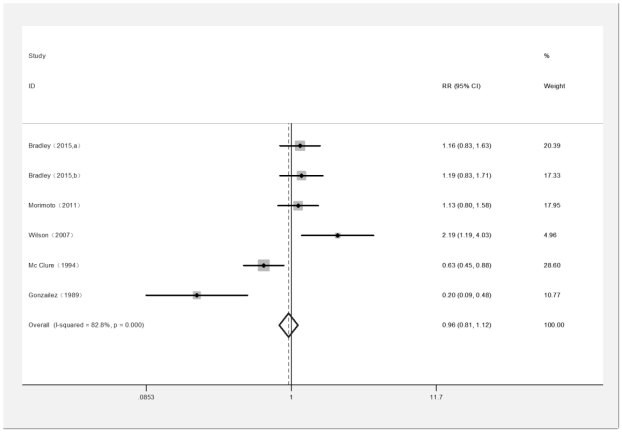

Figure 4. A forest plot of risk ratios and 95% confidence intervals for 6 records as-sessing the efficacy of *Escherichia coli* vaccines

Each dot in the funnel plot represents a set of data. Data with larger sample size were allocated to the higher position on the diagram. The funnel plot of *Staphylococcus aureus* vaccine was visually symmetrical (Figure 5), while the funnel plot of *Escherichia coli* vaccine was visually asymmetrical (Figure 6). The result of Egger's test was consistent with visual assessment, with p-value 0.642 and 0.614 respectively (Table 5,6).

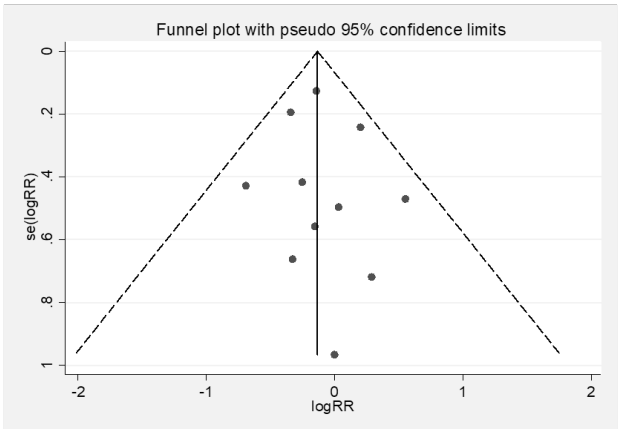

Figure 5. A funnel plot illustrating the deviation of meta-analysis assessing the efficacy of *Staphylococcus aureus* vaccines

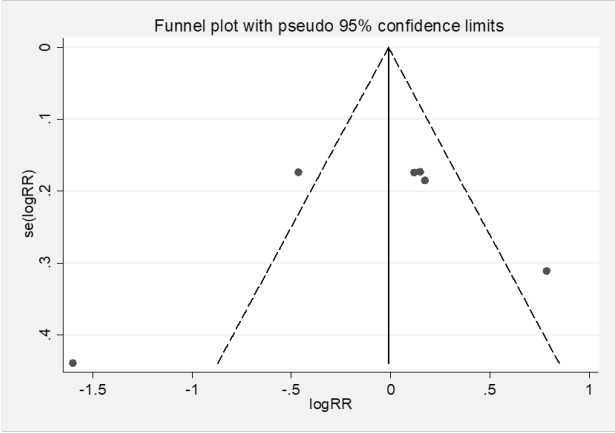

Figure 6. A funnel plot illustrating the deviation of meta-analysis assessing the efficacy of *Escherichia coli* vaccines

Based on the parameters set in the previous study, the incidence of mastitis in vaccinated group and control group was assumed as 0.125 and 0.25 respectively, the probability of testing type one error was assumed as 10%(α=0.1), the probability of testing type two error was assumed as 80%(1-β), therefore the μ_a and μ_b was 1.645 and 1.282. So the minimum sample size was 167.

The subgroup analysis revealed that sample size had no influence on the conclusion of the efficacy of the *Staphylococcus aureus* vaccine with RR 0.87(0.72, 1.07) and 0.86(0.72, 1.02) respectively (Figure 7). The sample size of all study in *Escherichia coli* vaccine group was larger than 167, thus subgroup analysis was not conducted.

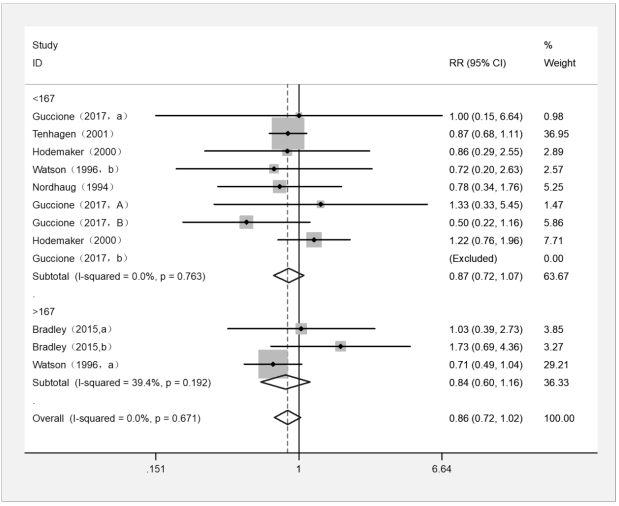

Figure 7. A forest plot of subgroup analysis assessing the influence of sample size

The result of sensitive analysis revealed that the conclusion of the efficacy of the *Staphylococcus aureus* vaccine was not stable. When two of twelve data sets were omitted, the pooling result converted to a positive conclusion with 95% CI excluding value 1 (Table 7). Contrarily, the conclusion of the efficacy of the *Escherichia coli* vaccine was stable for no conversion of the conclusion was detected (Table 8).

4. Discussion

The aim of this meta-analysis was to assess the efficacy of vaccine against mastitis caused by *Staphylococcus aureus* or *Escherichia coli* through pooling previous studies. The overall effect indicates that vaccination does not provide significant protection against bovine mastitis caused by *Staphylococcus aureus* or *Escherichia coli* with RR 0.86 at 95% CI $^{[0.72,1.02]}$ and 0.96 with at 95% CI $^{[0.81,1.12]}$ respectively, both showing the inclusion of the value 1 in the CI.

The results of quality assessment revealed half of 6 randomized controlled trials mentioned the "random" in the description, but failed to specify the method of random allocation, and compromised the quality of the study. One study allocated cows based on ear tags (odd number and even number was divided into two groups). Although this practice reduces the workload, it cannot be considered as an appropriate randomising method. The process of random allocation consists two main steps. The first step is the generation of random sequences. Multiple methods are available to achieve this goal, such as the random number table method and SAS. The generated random sequence is to be used as a random allocation scheme. The next step is to conceal allocation, and prevent personnel from consciously or unconsciously influencing the experiment outcome. Appropriate randomization can reduce the individual's impact on the results. For example, self-resolution is possible in mastitis, and will affect the evaluation of vaccine efficacy. However, this ability is different among individuals, and relevant factors include immune status and genetic resistance. Appropriate random allocation reduces the influence of these factors, and increases the accuracy of results. The results of a study without allocation concealment can be exaggerated by 30%-41% comparing to those with allocation concealment.

Some studies did not report whether the participants were blind to the treatment. Unlike human clinical trials, animals are inherently considered as blind participants under most circumstances, as a result, personnel are the only concern in this aspect. The blind method is implemented to ensure everyone involved in the experiment is unaware of the precise allocation plan, and preserves the impartiality of the evaluation. Pereira also pointed out in his systematic review that some trials may conceal true efficacy of vaccines due to the lack of double-blind measures [11]. This reminds the necessity of comprehensive design and detailed description regarding random allocation method

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in future studies.

It is also important to cover economic factor in future studies. The cost-profit analysis should cover the impact of withdraw period, productivity loss, and vaccination cost, for dairy industry is primarily profit driven. Ozsvari conducted a survey in a large-scale Hungarian dairy farm which initiated Startvac® application in 2010. The survey reported that the mastitis vaccination increased average annual profit by € 50.7 (decrease in loss-cost of vaccination) per cow between 2011 and 2014 [18]. Although Startvac® is not effective in reducing incidence, it may have a significant positive impact on reducing the quantity of discarded milk. Our meta-analysis suggested that the polyvalent inactive mastitis vaccine.

5. Conclusions

The results in this study revealed the efficacy of vaccine against mastitis caused by *Staphylococcus aureus* or *Escherichia coli*. The overall effect indicates that vaccination does not provide significant protection against bovine mastitis caused by *Staphylococcus aureus* or *Escherichia coli*.

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ARTICLE

A Comparative Investigation on Growth of Three Food Born Pathogenic Bacteria Inoculated with *Withania somnifera*: an Invitro Experimental Study

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ABSTRACT

Background: *Withania somnifera* (*WS*) is proposed as one of the alternatives instead of the antibiotic. This study is aimed to evaluate the inhibitory potency of enzymatic extract of the fruits of the *WS*. Methods: As an invitro experimental study, the growth rate of *Shigella dysenteriae, Salmonella typhimurium*, and *Escherichia coli* inoculated in different concentrations (25%, 12.5%, 6.25% and 3.125%) of the extract were assessed. A microtitre plate method was conducted. ANOVA was applied to identify statistical differences with *p*-value <0.05. Results: Different concentrations of extract, in comparison with control, declined the growth rate of all tested bacteria. All concentrations inhibited the growth of *S. typhimurium* (p<0.05). Compared to the microorganism control, effective concentration of the extract inhibiting the growth of *E. coli* was 12.5%, and 6.25%, while it was 12.5%, and 6.25% for *Sh. dysenteriae* (p<0.05). A dose-dependent response of *E. coli* was observed. The antibacterial activity of the extract tested was found mainly against *E. coli* and *Sh. dysenteriae*. The most resistant microorganism compared to *E. coli* and *Sh. dysenteriae* was *S. typhimurium* (*p*<0.05). 25% of the concentration of the extract showed the different inhibitory effect among three tested bacteria (*p*<0.05). Conclusions: The extract was labeled as an antibacterial agent against the representative of three foodborne bacteria, Invitro. The common effective concentrations of the extract (12.5, and 6.25%) is recommended for further research, as food additive, to remedy digestive ailments related to *E. coli*, *S. typhimurium* and *Sh. dysenteriae*.

1. Key Messages

The results of this study highlight the antibacterial effect of *Withania somnifera* (*WS*), a medicinal plant in Sistan and Baluchistan province, Iran, against *Shigella dysenteriae*, *Salmonella typhimurium*, and *Escherichia coli* (*E. coli*), as food born human/animal pathogens.

As a continuous work to our previous relevant study, the present study figures out the effect of different concentrations of crude enzymatic extract of *WS* berries against *E. coli*, *S. typhimurium* and *Sh. dysenteriae* growth, and also, register the local understanding of traditional medicines' use by residents in Sistan and Baluchistan.

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2. Introduction

Today, around 60% of anti-infective and antitumor medicaments, found in the market, composed of natural origin. [1] Medicinal herbs have fewer side effects, and are cheaper than chemical drugs, and also, easily available. [2] Statistics on the use of herbal medicines are significant in recent years. The World Health Organization revealed that about 4 billion people, now, use herbal medicine to treat illnesses. [3] Maybe, it is an unlikely explanation for many people that a variety of the chemical drugs in colorful packaging is the result of scrutiny on the effective elements of medicinal plants. This has led to form a widespread investigation by pharmaceutical companies on the healing properties of plants in different parts of the world. [3]

Designation of a national pharmacopeia, monographs of medicinal materials, standards and guidelines regarding medicinal herbs is suggested. [2] *WS* is considered as a medicinal plant in Sistan and Baluchestan province, Iran. [4,5] This plant is one of the vegetation of many cities of South of the province, including Saravan, Iranshahr, Sarbaz, Sib -o- Soran, Mehrestan, and Khaash (Figure 1). [6] It is widely distributed and different parts of *WS* are prescribed, empirically, in traditional medicine, in above locations, for the treatment of diarrhea, vomiting and hypertension. [6-8] Aqueous, ethanol, methanol, petrol, and chloroform extracts of leaves and roots of *WS* have been examined on *S. typhimurium*, *Sh. dysenteriae* and *E. coli*. Nevertheless, the effect of berries of *WS* is ambiguous. [9-11]

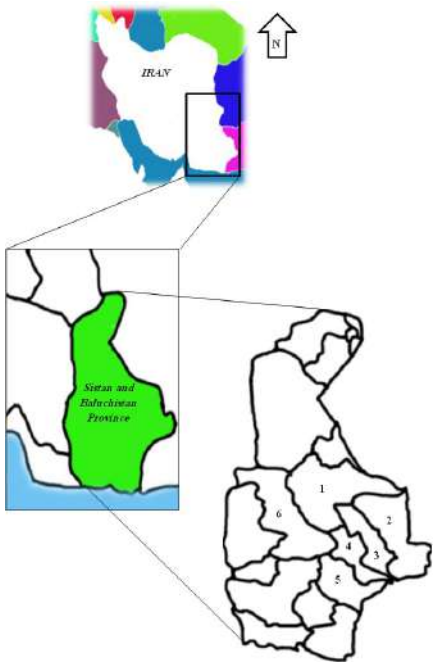

Figure 1. Cities of Sistan and Baluchistan province used for sampling. 1: Khaash, 2: Saravan, 3: Sib -o- Soran, 4: Mehrestan, 5: Sarbaz, 6: Iranshahr

Intestinal bacteria are divided into opportunistic pathogens like *Escherichia coli*, and primary pathogens including *Salmonella* and *Shigella* spp. They infect the digestive system of human and animal, lead to diarrhea and digestive system disorders. [12] In addition, they could act as food borne pathogen via consumption of contaminated water or meat. [13] Antibiotics are used to inhibit the growth of these bacteria and it results in the emergence and development of the resistance isolates. Hence, to discover new agents with distinct origin, as substitution for antibiotics, is logical, nowadays [2].

The present study is carried out to investigate the antimicrobial activity of different dilutions of extract of fruits of *WS*, using a microtitre plate method, against three commercially available bacterial strains, including *E. coli*, *S. typhimurium*, and *Sh. dysenteriae*, as representative of intestinal food borne gram-negative pathogens in Iran.

3. Materials and Methods

3.1 Bacterial Strains and Culture Media

E. coli (ATCC® 25922™; PTCC® 1399™), *S. enterica subsp. enterica* serovar *typhimurium* (ATCC® 14028™; PTCC® 1709™), and *Sh. dysenteriae* (PTCC® 1188™), were delivered from archive of Laboratory of Microbiology, Faculty of Veterinary Medicine (LMFVM), University of Zabol, Zabol, Sistan and Baluchistan, Iran, and used for the current study. Bacterial cultures were grown in Peptone Water Broth (PWB). Two hours culture of tested bacteria, amplified in 5 mL PWB, were adjusted to 0.5 McFarland standard (about 10^8 CFU, confirmed by plate colony count). [2]

3.2 Plant Material

WS were collected from cities of Sistan and Baluchistan province (Figure 1), Iran, at the markets, and at the homes of traditional healers during April - July 2017, and transferred to LMFVM. The identification of the plant was conducted entirely by Department of Plant Pathology, Faculty of Agriculture, University of Zabol, Iran.

3.3 Extract Preparation

Briefly, enzymatic extract of *WS* fruits was obtained by homogenization of 10 g of *WS* berries in 60mL of 85% NaCl for 24 hours via mild shaking, and then, centrifugation at 20,800 × g for 30 min at 4°C. The supernatant was filtered and applied for *invitro* study or stored at 4°C for next steps. [6, 14] Identification and quantification was performed, considering total protein concentration of enzymatic extract via Bradford method. [15]

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3.4 Formulation of Different Concentration of Extract

Five dilutions of extract (0%, 25%, 12.5%, 6.25%, and 3.125%) were prepared in this study.

3.5 Antimicrobial Assay

100 µL of adjusted culture to 0.5 McFarland standard was distributed into flat-bottomed 96-well microtiter plates and mixed with 100 µL of different concentration of *WS* berries extract. As microorganism control, 100 µL of PWB was mixed with 100 µL of adjusted culture to 0.5 McFarland standard. In addition, culture medium control consist of 100 µL of PWB mixed with 100 µL of PWB was included. Plates were incubated at 37 °C, and the growth, as Optical Density (OD), was evaluated using a micro-plate reader (Stat fax-2100, UK), set at 490 nm, at time 0 and 24 hours after incubation. All bioassays were carried out in triplicates. The antimicrobial assay was carried out for *E. coli*, *S. typhimurium* and *Sh. dysenteriae*, individually. [2]

3.6 Statistical Analysis

The growth curve of tested bacterium, based on OD, for different concentration of *WS*, and control, was computed and the slope value was calculated via regression coefficient (B). In better words, the increases and decreases in growth rate were calculated by slope of trend lines during tow measurements (0 and 24) using Microsoft Excel [16]. More negative slopes value demonstrated more inhibition. Statistical Package for the Social Sciences (SPSS) was applied to analyze statistics via ANOVA and to identify differences with p-value <0.05 [16].

4. Results and Discussion

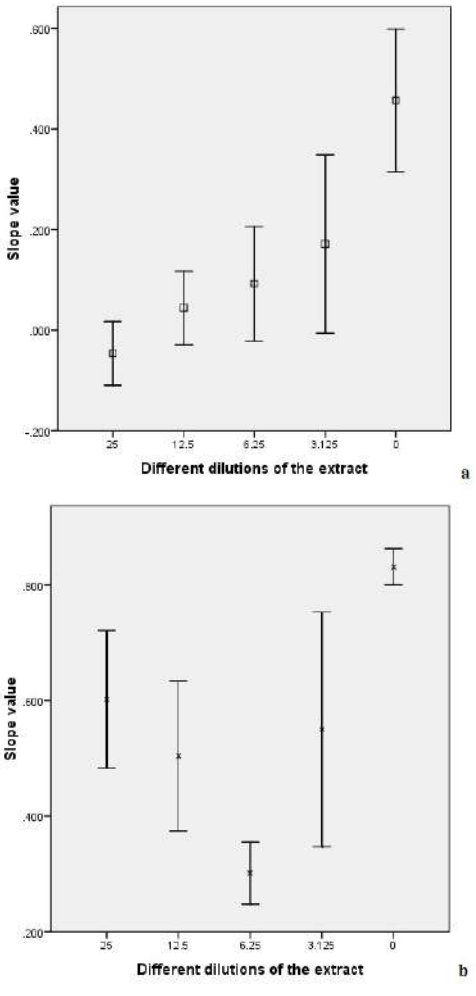
High interest in traditional medicine has been declared by WHO. [3] In Sistan and Baluchistan, one of the biggest provinces of Iran, primary health care have been mediated via traditional medicines. [5] Bacterial infections and inflammation are among the sicknesses treated by traditional healers in study area. Few investigations regarding traditional medicine have been accomplished in South-East of Iran, Sistan and Baluchistan, one of the old provinces of Iran. [2]

Regional healers for treatment of diarrhea administrate *WS* in Sistan and Baluchistan. Based on our literature review no study about *WS* was performed in the study area [4].

There are literally many published scientific papers from around the globe describing the antimicrobial activities of *WS* extracts and its chemical constituents, but none of them deals with fruit extract of *WS*. [17-19]

As a continuous work to our previous relevant study, [2] the present study is performed to figure out the effect of different concentrations of crude enzymatic extract of *WS* berries against *E. coli*, *S. typhimurium* and *Sh. dysenteriae* growth, and also, to register the local understanding of traditional medicines' use by residents in Sistan and Baluchistan.

The growth rate of *E. coli*, *S. typhimurium* and *Sh. dysenteriae* were assessed via the calculation of slope line trend. All microorganisms tested were found to be susceptive to the extract and their growth was inhibited compared to their control (Figures 2). The lowest growth rate, with significant difference (p<0.05), was observed at 25%, 12.5% and 6.25% of the concentrations of the extract for *E. coli* (Figures 2a). Moreover, based on figure 2b, all concentrations of the extract showed inhibitory effect, with statistical significant difference (p<0.05), on the growth of *S. typhimurium* compared to the microorganism control. Finally, two concentrations of the extract, including 12.5% and 6.25%, decreased the growth rate of *Sh. dysenteriae*, statistically significant (p<0.05), compared to its control (Figure 2c). In addition, as can be seen in figure 2, notably (p<0.05), 12.5% and 6.25% of concentrations of the crude enzymatic extract of *WS* berries showed the best inhibitory effect for three tested bacteria.



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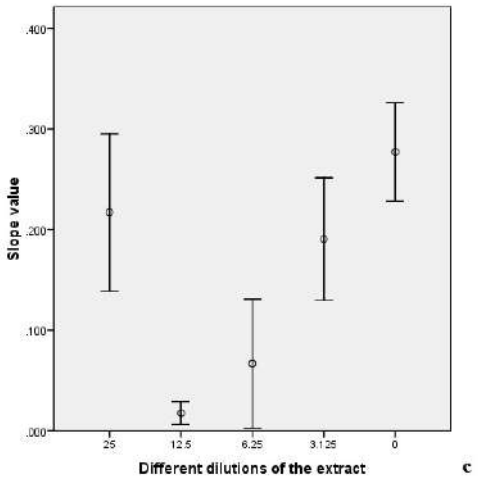

Figure 2. The effect of different dilutions of extract on *E. coli* (a); *S. typhimurium* (b); *Sh. dysenteriae* (c); the dots show slope value of trend lines and error bars indicate 95% confidence of interval of Mean. More negative slopes value demonstrated more inhibition

As a first report, (1) the inhibitory effect of *WS*, particularly crude enzymatic extract of its berries, on *Sh. dysenteriae* is reported, (2) comparison of inhibitory effect of the extract on bacterial growth is performed in Iran by a simple and user-friendly method, (3) the representative of three food borne pathogens were selected, and the most effective concentration of the extract, among examined concentrations is introduced for pathogens, individually.

These results are well comparable to the in vitro antibacterial activities of crude extracts of *WS* against human pathogenic bacteria such as *S. typhimurium* and *E. coli*. [11] The method used in the present work and the component of the plant are different. Furthermore, strain TA100 of *S. typhimurium* and DH5α strain of *E. coli* was investigated by Arora et al. [11]

The present study deals with only crude enzymatic extract of fruit of *WS*. It is suggested to specify and contemplate purifying all the fractions and check the antibacterial activity of individual compounds. Checking the efficiency of individual compounds is more desirable and warranted to increase importance of the present investigation. Antimicrobial properties of a non-toxic glycoprotein (*WS*G) extracted from the root tubers of *WS* is documented. Evidences clearly indicate that *WS*G is a protease inhibitor and exertion of antifungal activity could be due to its protease inhibitory nature. Moreover, findings ruled out the possibility that *WS*G is a ribonuclease/ deoxyribonuclease [10]. This kind of glycoprotein may be found to accomplish the antimicrobial properties of the fruit of *WS* that requires more investigation.

Antimicrobial activity of leaf extract of *WS* against antibiotic resistant *Staphylococcus aureus* was assessed and concluded that ethanol extract of *WS* leaf might be exploited as natural drug for the treatment of several infectious diseases caused by this pathogen [20]. A study evaluated the antibacterial activity of aqueous and alcoholic extracts of root and leaves of *WS* against pathogenic bacteria including *S. typhimurium* ATCC 23564, *E. coli* K-12 DSM 4060 and *Staphylococcus aureus* ATCC 9144 by in vitro agar gel diffusion method and it was found to possess strong antibacterial activity against mentioned bacteria. [21] According to the antimicrobial properties of the plant, our findings in the current study was not different from those of Bokaeian and Saeidi [20] and Owais et al [21].

A study investigated the antibacterial activity of *WS* root (*WS*R) against *E. coli* O78. The turbidity optical density was measured. The results revealed that the maximum inhibition of bacterial growth was observed at 1:8 dilution of *WS*R extract. The authors concluded that *WS*R possessed good antibacterial activity [22]. It is consistent with our findings which was observed the lowest growth rate of *E. coli*, at 25%, 12.5% and 6.25% of concentrations of the extract ,with significant difference ($p<0.05$).

As shown in Figure 2a, the slope values, for the extract with the E.coli showed a dose-dependent decrease with the increase in the concentrations of extract, while, it was not seen for both *S. typhimurium* and *Sh. dysenteriae* (Figures 2b and 2c). One study found that *WS* caused dose-dependent suppression of α2-macroglobulin (an indicator for anti-inflammatory drugs) in the serum of rats inflamed by injection of carrageenan suspension. [23] Furthuremore, a study revealed that the aqueous extract of *WS*R inhibited the growth of bacteria in dose-dependent manner. [21] Also, in present study, as detailed information, the dose-dependent response of the extract of a *WS* component against *E. coli* has been shown.

Due to the antibacterial activity of the extract, the results presented in this paper documented that tested plant used by the healers in Sistan and Baluchistan for treatment of diarrhea, may act toward diarrheal diseases believed to be of bacterial origin. These facts support the medicinal value of *WS* and suggest that it could be the new sources of antibacterial therapies.

Again, it is worthwhile to note that according to our literature review, the present study is the first report related to the effect of different concentrations of enzymatic extract of *WS* fruits on three important intestinal food borne gram-negative pathogens including *E. coli*, *S. typhimurium*, and *Sh. dysenteriae*. Among tested bacteria, different concentrations of *WS*, in term of inhibitory effect, are compared, statistically. All microorganisms tested were found

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to be susceptive to the extract. Different concentration of the extract, showed various extent of growth inhibition among tested bacteria, individually (Fig 3). The power of the inhibition of the extract among three bacteria, based on the concentration is compared in table 1. As can be seen in table 1, notably ($p<0.05$), 25% of concentration of the extract showed the different inhibitory effect among three tested bacteria (table 1, Fig 3), indicating that the inhibitory effect of the 25% of concentration of the extract could be species-specific among bacterial population. The decreasing order of the growth rate, with significant difference, in confronting with the extract were as *E. coli* > *Sh. dysenteriae* > *S. typhimurium* (Fig 3). The results clearly indicated that *E. coli* and *Sh. dysenteriae* are the bacteria with the highest sensitivity to the extract compared to *S. typhimurium* (Fig 3). There are studies that report selective antibacterial activity of extract of *WS* inhibiting the growth of bacteria, which is consistent with our results. [24]

Table1. Comparison of the inhibitory effect of different concentration of the extract on the growth of tested bacteria

tested bacteria	Concentration (%)			
	25	12.5	6.25	3.125
E. coli vs *S. typhimurium*	■	■	■	
E. coli vs *Sh. dysenteriae*	■		■	
S. typhimurium vs *Sh. dysenteriae*	■	■		
E. coli vs *Sh. dysenteriae*	■			
The comparison that are significantly different ($p<0.05$) from each other at the same concentration point indicated by ■.				

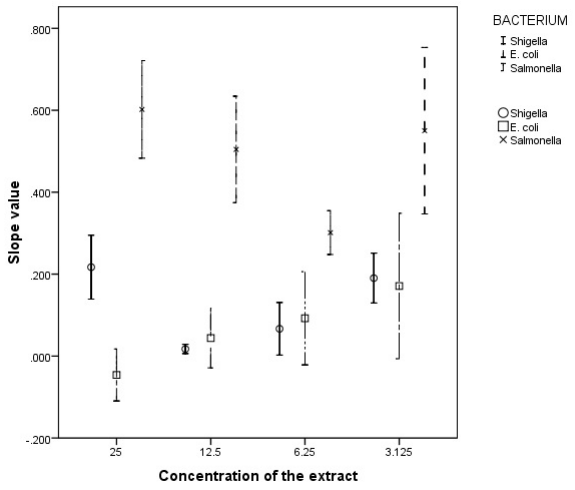

Figure 3. The effect of different dilutions of extract on three tested bacteria; Dots show slope value of trend lines and 95% confidence of interval of Mean are presented by error bars. More negative slopes value demonstrated more inhibition

A study screened the crude extracts of different parts of *WS* including unripen fruit; ripen fruit, and calyx, for their antimicrobial activity invitro against Bacillus subtilis, Pseudomonas aeruginosa, and Enterobactor aerogens by disc diffusion assay. Chloroform extract of calyx of *WS* showed highest activity against *Bacillus subtilis*. [25] Our study was the first report to evaluate the effect of crude fruit extract on *E. coli*, *S. typhimurium* and *Sh. dysenteriae* and the results obtained in the present study may be due to the genera of tested bacteria and methodology compared to the study conducted by Singariya et al. [25]

Antimicrobial activity of crude acetone extract from the aerial part of *WS* was tested in vitro against six pathogenic bacteria, using disk diffusion method, in comparison with gastrointestinal microbiota, and the results suggested that *WS* could act as an effective antibacterial agent against human pathogenic bacteria with lowered harmful effect on bifidobacteria [9]. The examination of different part of *WS* with different methodology in the present work may justify the difference of our observed data compared to the findings of Halamova et al [9].

Identification, extraction, and purification of more than 35 chemical constituents of *WS* has been widely studied. Alkaloids, steroidal lactones, saponins, and withanolides have been introduced as the biologically active chemical compounds of *WS*. [19] The structural and non- structural proteins of examined bacteria, in comparison with other tested bacteria, may play a role to super-induce/prevent the inhibitory effect of the extract [26].

The results of the present study portray the prospect of using *WS* as a substitution for antibiotics in the bacteriology. It is important to demonstrate scientifically that the remedies employed in folk medicine are indeed therapeutically active and therefore, potentially active compounds must be isolated from tested plant and according to our outcomes, *WS* would be interesting candidates for future research regarding to *E. coli*, *S. typhimurium* and *Sh. dysenteriae*. Bear in mind that the mutagenic and/or toxic effects of *WS* is still ill-defined and could act as growth depressor. Further studies on the mutagenesis/toxicity of the plant must be employed, as well as its application in often complex traditional mixtures. It would allow to elucidate possible candidates for future development of antimicrobial agents.

It should not be out of mind that minimum inhibitory concentration, double disc diffusion test, standard susceptibility breakpoints and resistance cut-off breakpoints for this plant fruit extracts need further investigation.

Studies showed that fruits extract of *WS* possesses good radical scavenging activity. [27] It is, also, reported that synthetic oxidants presenting in both food and drugs

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can lead to undesirable health effects. With the latest trend, crude fruit extracts of *WS* as antioxidants have been potentially proposed to add by many food technologists to increase the nutrient values. The use of natural antibacterials for treating diseases, and as food additives, have better consumer acceptability and a trend over the use of the available synthetic products. [27]. Our result, as a research of quantification of the antibacterial activities of the *WS*, indicated that two concentration of the extract, including 12.5% and 6.25%, play significant role to decrease growth rate of three important food borne pathogens, including *E. coli*, *S. typhimurium* and *Sh. dysenteriae*. Our result is important since the information on the antibacterial properties of *WS* against *E. coli*, *S. typhimurium* and *Sh. dysenteriae* is available prior to incorporating them into food products. The findings may be used as a fundament for further experiment in food technologies to control diseases. It is notable that harmful adverse effect on beneficial human microbiota, regarding to plant extracts and compounds, need more investigation. On the other hand, vegetarianism can also lead to increase the demand for substitution of plant material instead of chemical drugs, especially antibiotics. Through this investigation, we have shown that all dilutions of extract exhibit antibacterial activities against three tested bacteria, proposed a good potential to be used in therapeutics. The results presented in this report will also provide a suitable guide in choosing dilutions of extracts by the medical practitioners as natural antibacterial treating and controlling diseases.

In sum, this paper reports and establishes a scientific basis for the therapeutic use of *WS* as an antibacterial agent against three food borne pathogens. This experiment reveals and proposes the effective dilutions of crude enzymatic extract of *WS* berries against the growth of *E. coli* (25%, 12.5%), *S. typhimurium* (25%, 12.5%, 6.25%, 3.125%) and *Sh. dysenteriae* (12.5% and 6.25%), and also, it increases the local understanding of traditional medicines' use by residents in Sistan and Baluchistan. 12.5% and 6.25% dilutions of crude enzymatic extract of fruit of *WS* can be proposed for further research, as food additive to remedy ailments related to examined bacteria.

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Conflict of Interest

The authors declare no conflict of interest for all potential sources of bias, including affiliations, funding sources, and financial or management relationships.

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