ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

EVALUATION OF ANTIMICROBIAL AND IMMUNE MODULATORY EFFECT OF PHYTOGENIC HERBS IN BROILER CHICKEN

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EVALUATION OF ANTIMICROBIAL AND IMMUNE MODULATORY EFFECT OF PHYTOGENIC HERBS IN BROILER CHICKEN

A thesis submitted to the College of Veterinary Medicine, Addis Ababa University in partial fulfillment of the requirements for the degree of Masters of Veterinary Science in Veterinary Microbiology

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LIST OF ABBREVIATIONS

AMPs	Antimicrobial peptides
CRD	Completely randomized experimental design
ELISA	Enzyme linked immune sorbent assay
EOs	Essential oils
EUL	European union legislation
GIT	Gastrointestinal tract
GPAs	Growth promoting antibiotics
Hb	Hemoglobin
HI	Haemagglutination inhibition
IBD	Infectious Bursal Disease
iNOS	Inducible Nitric-Oxide Synthase
IEL	Intraepithelial Lymphocyte
NCDV	Newcastle Disease Vaccine
NGPs	Natural Growth Promoters
NK	Natural Killer Cells
NVI	National Veterinary Institute
OIE	Office of Infection des Epizootics
PFA	Phytogenic Feed Additives
RBC	Red Blood Cell
WBC	White Blood Cell

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ABSTRACT

Phytogenic feed additives have gained attention in livestock feeding following the ban of antibiotic growth promoters because, they are natural, less toxic, residue free and ideal feed additives in animal production. However, they are not scientifically well investigated and studies conducted so far, only forwarded their traditional use. This study was conducted in the College of Veterinary Medicine and Agriculture poultry farm of Addis Ababa University, Bishoftu campus from November 2019 up to April 2020 to determine the antimicrobial and an immune stimulatory effect of five herbs, at 1% inclusion on the basal broiler ration. For this study 378, a day-old broiler chicken were randomly assigned to six feeding trials (five treatments and one Control) each with three replicates containing 20 chicks based on a completely random design (CRD). The treatment groups were given basal diet mixed with 1% of one of the five herbs Thyme, Mint, Basil, Rosemary and Lemongrass. Representative chickens from each feeding trials were slaughtered at 21st and 42nd day so that cecal bacterial load and lymphoid organ weights were investigated. Serology was conducted to check for the antibody titer developed against NCD and IBD vaccine administered as challenge. As of the finding of this study, the lowest *E. coli* counts were seen on groups fed with Rosemary (5.3294$\pm$ 0.0717*)* followed by Lemon Grass (6.1269$\pm$ 0.0160). The highest *Lacto bacilli* counts were recorded in groups fed with Rosemary (10.375$\pm$0.0902) and Lemon grass (9.977$\pm$ 0.0161). Lemon grass, Rosemary and Thyme has shown the highest percentage mean Thymus index of (63.65%), (63.27%) and (63.25%) respectively. These findings suggest that herbs produced in Ethiopia could be used as PFA to minimize risks of infections with pathogenic bacteria. However, detail scientific investigation on their medicinal use and their combined formulation as feed additive is lacking. Therefore further studies which combine both *in-vitro* and *in-vivo* investigation should be conducted for better discovery of their medicinal effect beyond solely reporting their traditional use.

Keywords: Antimicrobial activity, Broiler chicks, Immune enhancing activity, Phytogenic herbs

1. INTRODUCTION

Growth promoting antibiotics (GPAs) are widely used to improve the production performance of meat producing animals, to modify the alimentary Micro biota, to boost productivity and for growth promotion (Huang and Lee, 2018).

The use of antibiotics as feed additives is hazardous due to cross-resistance and multiple resistances of pathogens (Schwarz *et al*., 2001). It has been documented that the use of antibiotics in chicken diets or drinking water has caused some undesirable results for chickens and consumers (Botsoglou *et al.,* 2004). Long-term use of those substances has led to development of drug-resistant microorganisms, reduced beneficial endogenous bacteria counts, the accumulation of antibiotic residues in animal products which is a threat to consumers' health and exerting a negative effect on the environment (Biernasiak *et al.,* 2010).

This caused their subsequent Ban by the European Union in 2006 & Following this ban, Phytogenic feed additives (PFA) have gained attention in livestock feeding being natural, less toxic, residue free and ideal feed additives in food animal production (Ibrahim et al., 2005).

PFA are classified as sensory and flavoring compounds, consisting mainly of plant extracts (essential oils, oleoresins, and flavonoids) and their active principles (Mountzouris *et al.,* 2011). The essential oils have been suggested to increase the growth performance , nutrient digestibility , and gut health (Jamroz *et al*., 2005 and Mc Reynolds *et al*., 2009) in poultry species.

The most widely used among these phytogenic feed additives are Thyme (*Thymus Vulgaris)*, Mint (*Mentha Spicata)*, Basil (*Ocimumbasilicum*), Rosemary (*Rosmarinus Officinalis*) and Lemongrass (*Cymbopogon schoenanthus).*Fresh and dried Rosemary leaves, whole or ground, are used as seasonings for soups, stews, sausages, meat, fish, and poultry, In Ethiopia it is used in the preparation of traditional food ingredients like

Berebere and Shiro etc. It's essential oil is used as an ingredient in various industries (Tigist *et al*., 2016).

Ethiopian Thyme (*Thymus vulgaris*), has the local name of '*Tossegne*' is one of the most favored salad and dessert dressings of all the regional herbs. The plant is common in meat dressing, vegetarian cuisines and even its droplets feature in herbal tea. Its greatest attribute perhaps is the marinating quality of adding flavor and aroma to popular dishes. The culinary uses of Thyme do not end at the plate. One of its best-known alternative uses is as a typical medicinal potion for overcoming stomachache, whooping cough and diarrhea. It has also been a very common remedy for reducing bedwetting in especially children due to its diuresis abilities that boost urination during the day (Edwards *et al*., 1976).

Ethiopia is world-renowned for its vibrant, herbal seasonings and infusions. Ethiopian Mint has more flavors concentrated in its leaves. It can be consumed as mint tea alone or added to other drinks to provide a minty kick. Among mint species, spearmint *(Mentha spicata L.)* is considered as an industrial crop as it is a source of essential oils enriched in certain monoterpenes like carvol, dihydrocarveole, dihydrocarveylacetate, menthol, menthone, caryophyllene, terpineol and cubebene which is widely used in food flavor, cosmetic and pharmaceutical industries (Edris *et al.,* 2003).

Sweet Basil *(Ocimumbasilicum L.)* is a branching herbaceous aromatic spice and medicinal plant that belong to the Lamiaceae family. Farmers of Ethiopia conventionally cultivate and use this crop for house consumption and provide for local market (Hassan *et al.,* 2007).

Lemon grass (*Tejsar*) is a perennial aromatic tropical grass that belongs to the family *Poaceae*. It is one of the 140 species that comprise the genus *Cymbopogon*. It is an aromatic grass that yields a wide array of essential oils useful in perfumery (Kumar *et al.,* 2009). Its name is derived from the typical lemon-like odor present in its shoot. Lemon grass has been used traditionally for cough, cold, rheumatism, lumbago, digestive and bladder problems, leprosy, and as a mouthwash for toothache and swollen gums. It has

also been used as a stimulant and diuretic (Hassan *et al.,* 2007). In Ethiopia, the leaf is used as a perfume, and in folk medicine for the treatment of heart, chest and stomach complaints (Asfaw and Demissew, 2009).

Several studies have identified only the traditional use of these natural products without detail experimentation to investigate their effect on growth performance, immunity, and on gut microbial population. This study therefore focused on evaluation of the antimicrobial and immune modulatory effect of these naturally occurring herbal (Basil, Rosemary, Thyme, Mint and Lemon Grass) feed additives in broilers.

Specific objectives:

- ✓ To evaluate the effect of herbs on gut microbial population.
- ✓ To evaluate the antibody titer among chicken kept under different feeding trials

2. LITERATURE REVIEW

2.1 Use of antibiotic as feed additives in poultry industry

The use of antibiotics to enhance growth and feed efficiency and reduce mortality in broiler production was introduced without rigorous testing as to efficacy some 50 years ago (Libby and Schaible, 1955). Few published studies since this time have provided evidence for a significant effect of GPAs on growth rates, feed conversion efficiency, or quality of the flock, which are the characteristics of importance in the economics of poultry production. It is appropriate to reconsider the premises for GPAs, since over the past 50 years many major changes in poultry production have been introduced, including selective breeding, controlled environments (lighting, temperature, and humidity), and supplementation of feeds with vitamins and minerals (Boyd, 2001). However, a selective pressure gave rise to bacteria resistant to antibiotics. This leaves scientists worried about the danger to human and animal health. Some strategies can be borrowed to reduce the use of antibiotics in chicken farms. Much research has been carried out to look for natural agents with similar beneficial effects of growth promoters (Mehdi *et al.*, 2018; Zhou *et al.*, 2007).

2.2 Shortcomings of antibiotic feed additives

A change in the intestinal micro biota of chickens can influence their immunity and their health. However, changes in the intestinal micro biota of chickens can be influenced by several factors. These factors include housing conditions, exposure to pathogens, diet composition and the presence of antibiotics in feed (Kers *et al.,* 2018).

The population of antibiotic-resistant bacteria, which was established during the time when antibiotics were routinely used, has survived from generation to generation for over 60 years even in the absence of antibiotic exposure (Wang *et al.*, 2008).

In addition to bio-resistance, antibiotics abuse has resulted in drug residues in animal products. Several antibiotics such as Penicillin, Tetracycline, Macrolide, Amino Glycoside and Amphenicol have been detected in feed(Diarra and Malouin, 2014).

2.3 Phytogenic feed additives

The term phytogenic feed additives were coined by an Austrian multinational feed additives company named Delacon, and was first introduced to the market in the 1980s. They are natural growth promoters used as feed additives, derived from herbs, spices or other plants. Botanicals or Phytobiotics, was also the name given to these products to describes plant-derived compounds incorporated in animal feed to improve productivity of livestock (Windisch *et al.*, 2008).

Phytogenics contain a cocktail of numerous different active principles (e.g. eugenol, cinnamaldehyde, Carvacrol or Thymol), which is responsible for the specific flavor or scent. They have also been used as flavoring agents, food preservatives and medicines since ancient times (Noonan, 2018).

Consisting a broad variety of extracts from plant parts, such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots, they are commonly regarded as favorable alternatives feed additives to antibiotic growth promoters in poultry production (Burt and Reinders, 2003). It was reported that these products of plant origin are natural, less toxic, residue free and ideal feed additives for animal when compared to synthetic antibiotics or inorganic chemicals. phytogenic substances have antimicrobial, antifungal anti-parasitic antiviral, anti-toxigenic and insecticidal properties (Burt and Reinders, 2003; Dingle and Alhassani, 2008).

2.4 Commonly used phytogenic compounds

Most common and frequently used herbs and spices for phytogenic feed additives in poultry production are oregano, thyme, garlic, horseradish, chili, cayenne, pepper, peppermint, cinnamon, anise, rosemary and sage. The beneficial properties of these compounds are due to their bioactive molecules such as Carvacrol, Thymol, Cineole, Linalool, Anethole, Allicin, Capsaicin, Allyl-isothiocyanate and Piperine (Yitbarek, 2015).

The addition of natural oregano essential oil can benefit both the lactating sow, as well as piglets. Oregano essential oil is well documented to offer support to animal health and performance as a result of the active compounds within it. Carvacrol and Thymol are two such compounds and these have been shown to demonstrate both antimicrobial and antioxidant functions in the animal. Oregano is known for its high content of essential oils and its antimicrobial effect, and the plant is a natural tool for reducing methane production in the rumen(Cabrera *et al.*, 2008).

Peppermint *(Menthapiperita,* also known as *M. balsamea* is a hybrid mint, a cross between water mint and spearmint. The plant, indigenous to Europe and the Middle East, is now widespread in cultivation in many regions of the world (Wang *et al*., 2008). Humankind has used plants for healing for many thousands of years, and it's from this tradition that the use of aromatic plant compounds in medicine begun. Oils were used in the embalming process, in medicine and in purification rituals. Essential oils are highly concentrated substances extracted from flowers, leaves, stems, roots, seeds, barks, resins, or fruit rinds (Yen *et al*., 2008). Mint oil has a number of uses it can be used to add a minty flavor to drinks, add mint flavor to food such as chocolates and icing and used in a number of natural applications from deterring ants to clearing up chest congestion. Making your own takes a few weeks, but is cheap and easy to do (Al-Taweil, 2014).

Lemongrass is a tropical perennial plant which yields the Cochin oil of commerce. The name of lemongrass is derived from the typical lemon-like odor of the essential oil

present in the shoot. *Cymbopogon citratus (C. citratus)* flourishes in sunny, warm, humid conditions of the tropics. Lemongrass will grow and produce average herbage and oil yields on highly saline soils(Farooqi and Sreeramu, 2001). In Nepal and India, lemongrass is traditionally used as a sedative, in addition to a treatment for fever, and an indigenous cure for infectious diseases. The herb has also been used as an external treatment for skin complaints like ringworm, athlete's foot and scabies. Because lemongrass has been known to control overactive oil glands, it can also be used as a toning astringent to cleanse oily skin and tighten pores. In some countries, it is used to reduce acne, pimples and blackheads. Lemongrass is also used as a treatment for lice and dandruff (Yoo *et al*., 2008). Through in vitro studies, the researchers examined the effect of citral, a molecule found in lemongrass, on both normal and cancerous cells. Citral, which is also found in lemon peels, is the substance that gives lemongrass its distinctive aroma and flavor. Compared to all other plants, lemongrass contains the highest amount of citral(Ohno *et al*., 2003).

Rosemary (*Rosmarinus officinalis* L.) is a spice and medicinal herb widely used around the world. Of the natural antioxidants, rosemary has been widely accepted as one of the spices with the highest antioxidant activity (Peng *et al*., 2005). Rosemary essential oil is also used as an antibacterial, antifungal and anticancer agent (Leal *et al*., 2003). Many compounds have been isolated from rosemary, including flavones, di-terpenes, steroids, and tri-terpenes. Of these, the antioxidant activity of rosemary extracts has been primarily related to two phenolic diterpenes: carnosic acid and carnosol (Frankel *et al*., 1996). The main compounds responsible for the antimicrobial activity are α-pinene, bornyl acetate, camphor and 1,8-cineole (Daferera *et al.*, 2000).

Table 1 commonly used phytogenic animal feed additives

Herbs /Spice	Latin Name	Plant Family	Main Constituent
Oregano	*Oreganum Vulgare*	Labiateae	Carvacol, Thymol
Thyme	*Thymus Vulgaris*	Labiateae	Thymol , Carvacol
Garlic	*Allium Sativum L*	Alliaceae, Liliaceae	Diallylsulfide, Alliciin, Alliin
Horseradish	*Armoracia Rusticana*	Brassicaceae	Allyl-Isothiocyanate
Chilli, Cayenne Pepper	*Capsicum Frutescens*	Solanaceae	Capsaicin
Pepper Mint	*Menthe Piperata*	Labiateae	Menthol, Carvacol
Cinnamon	*Cinnamonum Cassia*	Lauraceae	Cinnamaldehyde
Anise	*Pimpinella Anisum*	Apiaceae, Umbelliferae	Anethol

Source: (Windisch *et al.,* 2008).

2.5 The mechanisms of action of phytogenics

The exact mode of action and properties of phytogenics have not been fully understood, but are believed to be mostly related to the antimicrobial action, anti-inflammatory activity, and antioxidant effect of phytogenics. Additionally, phytogenics are often claimed to improve the feed flavor and palatability, which could lead to an increase in voluntary feed intake and growth performance (Windisch *et al.,* 2008). Phytogenic herbs, spices and their extracts can stimulate appetite and endogenous secretions such as enzymes or have antimicrobial, coccidiostatic or anthelmintic activities in monogastric animals (Wenk, 2003). The mechanisms of action and effects of phytogenics can further be explained in detail in the next sessions

2.6 Effects of phytogenics

The beneficial effects of herbs and spices in poultry production are primarily pertinent to improving the birds' ability to digest feed. Adding herbs to feed has an effect on digestive enzymes which results in an overall improvement in performance parameters such as weight gain and feed conversion. Better utilization of feed means less excretion, and this helps reduce environmental pressure, with the added benefit that these are natural

ingredients. They also offer several health benefits including immune stimulation, anti-bacterial, coccidiostatic, antiviral or anti-inflammatory activity and anti-oxidant properties (Groom *et al.*, 2007).

2.6.1 Growth-promoting effect

Mode of action of growth-promoting feed additives arises from relieving the host animals from immune defense stress during critical situations which increase the intestinal availability of essential nutrients for absorption, so that animals using these products grow better within the framework of their genetic potential. On the other hand, the mechanism of action of growth promoting feed additives arise from stabilizing feed hygiene and even more from beneficially affecting the ecosystem of gastrointestinal micro biota through controlling potential pathogens (Roth and Kirchgessner, 1998).

2.6.2 Antimicrobial effect

Phytogenic feed additives (PFA) are well known to exert antimicrobial actions in vitro against important pathogens, including fungi (Burt, 2004; Si *et al*., 2009). Phytogenic feed additives are more effective on Gram-positive bacteria compared to Gram-negative bacteria as of the report of most studies (Ceylan and Fung, 2004). This does not mean that the plant extracts are not active on Gram - bacteria, but the dosage should be higher (Burt, 2004). Some studies with broilers demonstrated in vivo antimicrobial efficacy of essential oils against Escherichia coli and *Clostridium perfringens* (Jamroz *et al*., 2005). Some phytogenic feed additives have been shown to act against Eimeria species after experimental challenge which suggests that phytogenic feed additives do also have anti parasitic effect besides their antifungal and anti bacterial effect (Giannenas *et al*., 2003).

2.6.3 Effects on intestinal health

Intestinal health problems often remain undiscovered because they are not necessarily reflected in clinical symptoms of a disease. Wet litter or liquid droppings are additional indicators of an upcoming digestive problem. Subsequently, clinical symptoms may

develop, and mortalities may increase. The reason for impaired intestinal health results from a stress situation in many cases, evoked, e.g. by high stocking densities, feed change, variations in ambient temperature and moisture, excessive growth of pathogens or technical errors of feed and water supply. A shift of the intestinal micro flora is a consequence; undesired microbes may multiply. Intestinal disorders usually cause a damage of the intestinal mucosa. The body's reaction includes an accelerated renewal of the damaged tissues. This process is complex and required additional energy. in other words, intestinal diseases go to the expense of performance (W. Windisch *et al.,* 2008).

Phytogenic substances reduce the microbial pressure and stabilize intestinal health. Nutrients that are supplied in the feed are absorbed by means of the villi, which loom into the interior of the intestine. Optimal functioning of the villi is essential for high performances. In broiler trials, it was seen that supplementation of feed with a PFA increased villus length, hence resulting in an increased capacity for nutrient absorption. The incorporation of phytogenic feed additives in poultry diets can significantly contribute to intestinal health and, hence, profitability in poultry production (Steiner, 2010).

2.6.4 Immune-stimulant effect

Macrophages play a crucial role in immunity against microbes by rapidly recognizing and phagocytosing pathogens. Moreover, macrophages activate antimicrobial effectors such as: inducible nitric-oxide synthase (iNOS) and cationic antimicrobial peptides (AMPs) to contain and clear pathogens. Increases in phagocytic activity can be elicited by saponins, as suggested by a previous study showing that saponin from Ophiopogon japonicas had marked macrophage-modulating activity represented by promotion of phagocytic capacity as well as increased macrophage viability rate, NO production and interleukin-1 release (Lee *et al.*, 2016).

Essential oils could change the lymphocyte distribution in the gut. Since a cross talk between gut Microbiota and the mucosal immune system is beneficial for a mutual growth and survival (Purchiaroni *et al.*, 2013), essential oils could leverage the

development and function of the gut immune system via modifying the gut micro flora. A mixture of carvacrol, cinnamaldehyde, and capsicum oleoresin decreased the population of intraepithelial lymphocyte (IEL) in jejunum and ileum, but increased lymphocyte in the lamina propria of early-weaned pigs (Manzanilla *et al*., 2006). Effects of some popular phytogenic feed additives on the immune response are reviewed here under Table 2.

Table 2: Effects of popularly used phytogenics on the immune response

Phytogenic materials	Species of experimental animals	Supplemented form	Effective dosages	Bioactive components	Enhanced performance/production	Immune responses	References
Echinacea purpurea L.	Broilers	Aerial part powder	5 and 10 g/kg diet		AWG, FI, and FCR	Higher antibody titers against SRBC and NDV	(Landy *et al.,* 2011)
	Laying hens and pigs	Pressed juice	0.25 mL/kg $BW^{0.75}$		No effect	Higher count of lymphocytes and NDV antibodies in the blood	(Böhmer *et al.,* 2009)
	Broilers	Aqueous extract	1 mL/L drinking water		No effect	Higher antibody titer against SRBC	(Rahimi *et al.,* 2011)
	Grower/finishing pigs	Cobs or pressed juice	1.5% cobs in the diet or 4–6 mL juice per day		FCR	Higher erysipelas antibody	(Maass *et al.,* 2005)
Oregano	Broilers	Essential oil	300 ppm in the diet		AWG and FI	Higher secondary antibody titer and IgG titer, lowered H/L ratio	(Mohiti-Asli and Ghanaatparast-Rashti, 2017)
	Broilers	Essential oil	50 and 100 ppm in drinking water	Carvacrol and thymol	BW	Higher antibody titer against NDV and avian influenza virus	(Galal *et al.,* 2016)
	Sows	Essential oil	250 ppm in the diet	Carvacrol and thymol	Energy intake	Higher thymus lymphocytes	(Ariza-Nieto *et al.,* 2011)
	Broilers	Essential oil	60–200 ppm in the diet	Carvacrol and thymol	AWG and FCR	Higher total antibody and IgG antibody, lowered H/L ratio	(Hashemipour *et al.,* 2013)
Cinnamon	Broilers	Powder	4 and 8 g/kg in the diet		FCR	Higher lymphocytes proportion	(Najafi and Taherpour, 2014)
	Broilers	Oil extract	100 and 200 ppm in the diet		WG, FI, and FCR	Lowered H/L ratio, higher white blood cells	(Al-Kassie, 2009)
	Broilers	Powder	5 g/L in the drinking water		Negative effect on BW	Higher antibody titer against NDV	(Sadeghi *et al.,* 2012)
Turmeric (*Curcuma longa*)	Broilers	Rhizome powder	2.5, 5, and 7.5 g/kg diet		Not detected	Higher IgA, IgG, and IgM levels, and decreased ratio of monocytes	(Emadi and Kermanshahi, 2007)

Continued page

Table 2 (continued)

Photogenic materials	Species of experimental animals	Supplemented form	Effective dosages	Bioactive components	Enhanced performance /production	Immune responses	References
	Broilers	Rhizome powder	2 g/kg diet		AWG and FCR	Higher total secondary antibody titer, and decreased H/L ratio	(Akhavan-Salamat and Ghasemi, 2016)
	Broilers	Dried rhizome extract	35 mg/kg diet		BW	Higher antibody level against Eimeria microneme protein	(Kim *et al.,* 2005)
Thyme	Broilers	Oil extract	100 and 200 ppm in the diet		AWG, FI, and FCR	Lowered H/L ratio, higher white blood cells	(Al-Kassie, 2009)
	Broilers	Essential oil	0.1% in the deit		Not detected	Higher white blood cells and decreased H/L ratio	(Khaksar *et al.,* 2012)
		Broilers	Essential oil	0.02% in the diet		FCR	Higher primary antibody titer

BW, body weight; AWG, average weight gain; FI, feed intake; FCR, feed conversion ratio; SRBC, sheep red blood cell; NDV, Newcastle disease virus; Ig, immunoglobulin; H/L ratio, heterophil to lymphocyte ratio.

3. MATERIALS AND METHODS

3.1 Study area

The experiment was conducted in the College of Veterinary Medicine and Agriculture poultry farm of Addis Ababa University, Bishoftu campus from November 2019 up to April 2020. Bishoftu town is located in the central high lands of Ethiopia at 47 km Southeast of Addis Ababa, the capital city of Ethiopia. The town is located at $8^o45'$ N longitude and $38^o59'$ E latitude at an altitude of 1880 m.a.s.l. It has an average annual rainfall of 1150 mm of which 84% falls down during the long raining season that extends from June to September, and the remaining during the short rainy season that extends from March to May. The mean annual minimum and maximum temperatures are 8.5 and 30.7 C, respectively, and the mean relative humidity is 61.3% (NMSA, 2010).

3.2 Experimental design and animals management

A completely randomized experimental design (CRD) was followed throughout the study period to evaluate the effect of phytogenic herbs on broiler chickens. Three hundred seventy eight (378) day old broiler chicks were purchased from commercial hatchery and reared in a wire-mesh and wood partitioned deep litter floor housing system (1.2m x 1.8m). Before their arrival, feeding troughs and watering equipment were thoroughly cleaned, disinfected against pathogens. On arrival, chicks were checked for any abnormalities and only the healthy ones were stocked together for the first five days, in order to encourage feed and water intake and accustom the new environment. All chicks were given standard starter chick diet for the first 5 days. On the 5th day, 5% (18 randomly selected chicks) were sacrificed to collect blood sample to have base line immune related data. The rest three hundred sixty (360) chicks were weighed and randomly assigned to one of six different feeding trials (1control and 5 treatments) each with three replicates containing 20 chicks based on a CRD. Chicks with extremely high and low weight were rejected and apparently healthy chicks were penned (see the design on appendix 1)

The room was well heated at a constant temperature of 32°C two days prior to their arrival and then was gradually be reduced after a week and then per week by 3°C until a final temperature of 20°C is reached on day 28 and then kept constant. Chicks of control group were fed basal diet which meet their nutrient requirements respective to their ages (i.e. chicks starter diet from day 1-10, grower diet from day 11-30 and finisher diet from day 31-42 purchased from suppliers) provided in mash form throughout the study time. The treatment groups were fed on Basal diet mixed with 1% of one of the five herbs Thyme (*Thymus vulgaris)*, Mint (*Mentha spicata)*, Basil (*Ocimum basilicum*), Rosemary (*Rosmarinus officinalis*) and Lemongrass (*Cymbopogon schoenanthus))* prepared in powder form as treatment.

Standard bio-security protocol was employed throughout the entire experimental period and disinfectant (Biosafe) was placed at the gate of the house for workers to deep in their feet before they enter into the house for prevention of disease introduction. All chicks were vaccinated against common broilers diseases as per the recommended local vaccination schedule/program of the hatchery and any abnormality or mortality occurring on the experimental units without the control of the researcher, were recorded.

3.3 Preparation of experimental herb powder

The treatment plants were purchased from Green Mark Herbs PLC., (Horticulture and Spice Farm, Hawassa) to incorporate in the diets of broiler chickens as phytogenic feed additives. The herbs were washed with tap water and then dried under the shade for three days. The dried leaves were prepared in powder form, sealed in plastic bags labeled for identification and stored at appropriate condition to be mixed later, in 1% inclusion to the basal diet as experimental rations. All kinds of feed additives used in the experiment were homogenously mixed with the broiler ration.

3.4 Variables to be measured

3.4.1 Evaluation of effect on gut bacterial load

Sample collection: samples (cecal contents and cloacal swabs) were collected from all the six experimental groups (six per group) on the 10th day after assigning them to their respective treatment groups, coded, and processed for the isolation and identification of enteric bacteria and enumeration of selected bacterial (pathogenic and normal flora), such as: *E. coli*, *lactobacilli* and other members of Enterobacteriaecea.

Isolation and identification of enteric bacteria: Cultural, morphological and biochemical characteristics were studied for identification of the bacteria. Gram's staining was performed to study the morphology and staining characteristics of bacteria, biochemical tests, such as catalase, MR, VP, and indole tests, were performed as per the standard methods (Cheesbrough, 1985). Isolation and identification of *Salmonella* was conducted based on ISO 6579: 2002 (Pavic *et al*., 2010). Accordingly, four successive stages were followed for the isolation and identification of *Salmonella.*

Pre-enrichment in non-selective liquid medium: cloacal swab was inoculated on Buffered peptone water at ambient temperature, and then incubated at 37 °C for 18 hours. Enrichment in/on selective media: Rappaport-Vassiliadis medium with soya (RVS broth) was inoculated with the culture obtained from pre-enrichment step which is 1ml of pre-enriched suspension in 10ml of RVS broth. The RVS broth was incubated at 41.5 °C for 24 hr.

Plating out on selective solid media: From the cultures obtained in enrichment stage, Xylose Lysine Deoxycholate agar (XLDagar) was inoculated. The XLD agar was incubated at 37 °C and examined after 24 h. XLD Agar is a moderately selective and differential medium for the isolation and differentiation of gram-negative enteric pathogens (*Salmonella* and *Shigella)* from clinical and non-clinical specimens. The cultured bacterial sample appeared as red colonies with black center. Besides primary isolation and characterization, secondary (biochemical characterization) tests were also undertaken so that upon culturing on TSI (Triple sugar iron agar) media, different

colonies were identified based on their properties to ferment one or two or all of the three sugars. Indole test, motility test, Gram Staining and other biochemical tests like Catalase, KOH and others also were undertaken consequently. Colonies of presumptive *Salmonella* were sub cultured and their identities were confirmed by means of appropriate biochemical tests (See annex 5).

For *E. coli* and coli forms: cloacal swab samples were transported in buffered peptone water and incubated at 37°c for 18hrs. A loop full suspension was transferred to nutrient broth. After overnight incubation at 37°c, the suspension was streaked on MacConkey agar plate and incubated at 37°c for 24hr. Lactose-fermenter and non-lactose fermenter was appreciated, their colony morphologies characterized and separately sub cultured on nutrient agar for further analysis. Pink colonies from MacConkey agar (lactose fermenter) were subcultured on Eosin Methylene Blue (EMB) agar. After overnight incubation, colonies with a characteristic metallic sheen were obtained (See annex 5).

For *Lactobacilli*: cloacal swab samples were dipped, and contents were thoroughly mixed in *de Man's Rogossa Sharpes (MRS) broth*. After overnight incubation, a loop full of suspension was streaked on MRS agar and incubated at 37 ºC for 72 hours in an anaerobic (micro-aerobic) environment using anaerobic jar with burning candle inside (See annex 5). Determination of *Lactobacilli* was performed according to their morphological, cultural and biochemical characteristics by the procedures described in the Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986).

After incubation on MRS agar for 24 h, isolates forming round, creamy white colonies were initially examined for Gram staining and catalase production. Then cell morphology and colony characteristics on MRS agar were tested. Gas production from glucose was assessed by inoculation of cultures into 5 ml MRS broth containing inverted Durham tubes and incubating at 35°C for 2 days.

Bacterial enumeration: On day 21 and 42 of the experiment, 3 birds per experimental group were randomly selected and slaughtered in a clean slaughter room, their viscera

were exposed and fresh Cecal contents were aseptically collected, ligated and carefully placed on sterile plastic bags for bacteriological assays (to enumerate (determine the colony forming units (CFU/ML) of the cecal bacterial load). The fresh contents were gently squeezed and pooled per treatment group on sterilized 25-ml screw cupped tubes. After vortexing the pooled content, 1 milliliter was transferred by a micropipettor to 9 milliliters of buffered peptone water, and the solution was shaken. Then, 1 ml of solution from tube number one was again transferred to the next tube (tube number two) and this operation was conducted up to tube number eight, and a dilution series was prepared within each treatment group from 10^{-1} to 10^{-8} (Mountzouris *et al.,* 2011) (See annex 5).

After vortexing of each dilution tubes, 20µl of each diluted samples was plated using spread plate technique on to three plates each, which were pre-labeled with their respective dilutions, treatment group code, name of the agar and the date plated. Bile Esculin Azide Agar, Violate Red Bile Agar and MRS agar were used for enumeration of the *Enterococci*, *Escherichia coli* and *Lactobacilli* respectively (Upadhaya and Kim, 2017). Bile Esculin Azide agar and Violate Red Bile Agar culture plates were incubated at 37 ºC for 24 hr. MRS agar plates were incubated at 37 ºC for 72 hours in a anaerobic (micro aerobic) environment using anaerobic jar with burning candle inside. *Enterococci, E. coli and Lactobacilli* colonizing the intestinal tract of chickens were enumerated among different feeding groups. Plates having countable colonies ranging from 30 to 300 colonies were selected out of the dilutions plated and the colony forming units were calculated based on those duplicate plate, such that the number of colonies were multiplied by the dilution factor and the reciprocal of the volume transferred (which is expressed in ml) (See annex 5).

The bacterial colony counts were calculated as colony forming units per milliliters of cecal digesta.

$$\text{Colony Forming Unit/ml} = \frac{\text{Average no of colonies}}{\text{dilutionfactor X volumeplated}}$$

3.4.2 Evaluation of effect on immunity

Eighteen (18) randomly selected (5% of the chicks) were sacrificed on day one of their age before vaccination, to collect serum sample to have base line immune data. Then the chickens were vaccinated for NCD (HB1 on day 1, NEW Lasota on day 12 and 24) and IBD on their 7th and 19th day of age. Blood collection was then carried out within interval, until the end of the experiment to get information on the development of immunity after a series vaccination.

After collection, the whole blood was left undisturbed for 30 minutes at ambient temperature to clot, the sample was then centrifuged at 1500 x g for 10 minutes and the serum was aliquoted to labeled cryovials and stored at -20°c. At the end of the experiment, all the preserved serum samples were shipped on ice box to National Veterinary institute (NVI) for serology. Haemagglutination Inhibition (HI) test (Sano and Ogawa, 2014) was performed (*See annex 6)* to determine the antibody titer developed against NCD and Indirect ELISA Test (*See annex 7)* for IBD (Al Suleiman *et al.,* 2020).

Since serological procedures usually involve serial twofold dilutions, the resulting titers are exponential functions of 2. Consequently, the simple expedient of logarithms to the base 2 (log_2) was used to express serological titration data. In a serological dilution series of 1:2, 1:4, 1:8 1:16 ... 1:2^n, the resulting log 2 values follow directly as 1, 2, 3, 4,…….n (Vennes *et al.*, 1957).

Lymphoid Organ Weights: Evaluation of lymphoid organs weight and determination of their weight to body weight ratio is the most used model to estimate protection rate given by vaccines against some diseases (Heckert *et al.,* 2002). At 21st and 42nd days of age, representative chicks from each pen were screened based on the average body weight of the pen and scarified. Immune related organs such as thymus, spleen and bursa of fabricious are exteriorized one after the other and weighed using sensitive balance in milligrams and the ratio of thymus, bursa, and spleen weight to body weight was calculated. The results were expressed for each experimental group as the arithmetic mean and standard deviation (Heckert *et al.,* 2002).

$$\text{Lymphoidorgan weight index} = \frac{\text{Weight (grams) X 100}}{\text{Body Weight (grams)}}$$

3.5 Ethical approval

Every procedure related to animal handing and their routine manipulations were conducted as of the guidelines of animal experimentation approved by the institutional animal ethics committee of Addis Ababa University, College of Veterinary Medicine and Agriculture.

3.6 Data analysis

The data generated, was subjected to statistical analysis technique which is appropriate for a completely randomized design. One-way analysis of variance (ANOVA) was used to test the effects of different dietary treatments using STATA software version 14. Significant difference among treatment means were checked at 5% probability level and results were presented as Mean ± SEM.

4. RESULTS

4.1 Cecal bacterial load

As of the finding of this study, the lowest *E. coli* counts were seen on groups fed at Rosemary (5.3294$\pm$ 0.07174*)* followed by Lemon Grass 6.1269$\pm$0.0160).Basil and Mint has also reduced the cecal load of *E. coli.* (See Table 3)

Almost all test plants have increased the cecal *Enterococcal* count compared to the Control group, with the highest count recorded during 21st day being Lemon grass (7.8192$\pm$0.05811) followed by Thyme (7.7007$\pm$0.06715). The mean logarithmic bacterial count was relatively similar on all test herbs though the highest count was recorded by Thyme (9.6124 $\pm$0.02248022) followed by Mint (90123 $\pm$0.3507) and Lemon grass (8.9977 $\pm$ 0.00925) compared to the lowest count on Control (7.3715 $\pm$ 0.08457).

The highest *Lactobacilli* counts were recorded in groups fed with rosemary(10.3755$\pm$ 0.09021) and lemon grass (9.9775$\pm$ 0.0161) followed by Mint (9.5038$\pm$0.0480). The lowest count recorded was seen on control group (7.2385$\pm$0.08834). As of the finding of this study statistically significant difference is seen ($P < 0.05$) on the mean logarithmic bacterial counts on all feeding trials. Nearly similar scenario of the results was seen on the 42nd day (2nd phase) investigation under each treatment groups except that the numbers significantly increased from the values formerly recorded during the 21st (first phase) investigation. Age wise analysis of the data at glance indicates that the count of normal bacterial flora has significantly increased due to the test herbs and the pathogenic bacteria counts were reduced in contrast, as of the record of the two phase investigation.

Table 3 Cecal bacterial load on day 21 and day 42

Feeding trial	**Day 21**			**Day 42**		
	Enterococci count	*Lactobacilli count*	*E. Coli count*	*Enterococci count*	*Lactobacilli count*	*E. Coli count*
Basil	7.6943614 ± 0.06505572	8.2345426 ± 0.06252373	7.9877159 ± 0.04733853	8.2503926 ± 0.13015021	10.025576 ± 0.21286035	7.6606438 ± 0.20100202
Lemon grass	7.8192446 ± 0.05811481	9.9775732 ± 0.0161665	6.1269566 ± 0.0160458	8.9977737 ± 0.00925977	12.6947873 ± 0.58586634	7.7467987 ± 0.23619506
Mint	7.6891308 ± 0.01758884	9.5038206 ± 0.0480812	7.2032701 ± 0.03843667	9.1230646 ± 0.35072963	10.246903 ± 0.12096508	8.4216164 ± 0.15536908
Rose	7.6869156 ± 0.07558564	10.3755433 ± 0.09021186	5.3294824 ± 0.07174139	8.3954099 ± 0.76095635	12.4621396 ± 0.30985645	7.0340929 ± 0.3619562
Thyme	7.7007003 ± 0.06715783	9.5542402 ± 0.05990166	8.1458509 ± 0.02194452	9.612493 ± 0.02248022	11.842468 ± 0.18478159	9.2251246 ± 0.63211571
Control	6.8047305 ± 0.11960174	7.2385606 ± 0.08834503	8.1687296 ± 0.01041091	7.3715588 ± 0.08457697	9.7963659 ± 0.47230191	8.0803078 ± 0.23779366
Value	*52.37*	*486.30*	*1753.05*	*10.32*	*9.67*	*10.24*
P-value	*0.0001*	*0.0000*	*0.0000*	*0.0066*	*0.0048*	*0.0040*

4.2 Effect on immunity

The current study was conducted to determine the effects on immunity of five different phytogenic feed additives in broiler chicken, with proper vaccination program against NCD and IBD. Experimental herbal additives that produce better immunity throughout the study period in response to NCD and IBD vaccines injections is screened via assessing the lymphoid organ mean weight indices as well as antibody titer detected by serological tests conducted on the serum sample of representative chickens collected during a course of study.

4.2.1 Lymphoid organ weight

The values of the body weight, thymus, bursa and spleen weight and their indices were summarized for each organ as (mean ±SD). As of the finding of this study, statistically significant difference is seen ($P < 0.05$) on the Lymphoid organ weight indices in all treatment groups on the second phase of study (day 42) though the result was statistically insignificant on the first phase (day 21), in spite of a large numerical difference on the weight indices. ($P > 0.05$) *(See Table 4).*

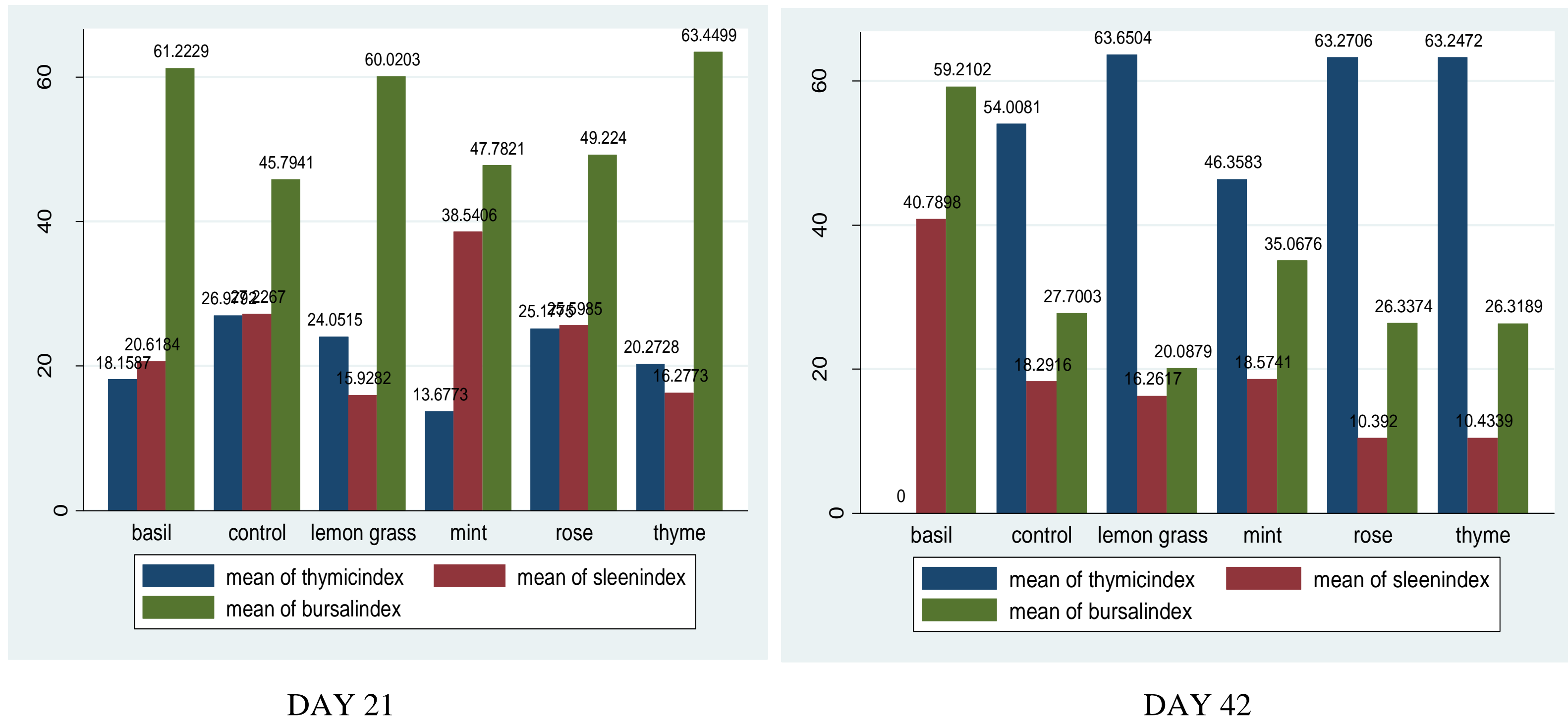
As is indicated on the bar chart (Figure 1), Lemon Grass, Rosemary and Thyme has shown the highest percentage (63.65%), (63.27%) and (63.25%) in mean Thymus index respectively than the other groups where, the lowest mean index being Mint (46.36%). This indicates that, Thymus and Rosemary group has shown relatively better performance on T-Cell production compared to the other groups and Control and there is statistically significant difference on their mean weight indices ($P < 0.05$).On the other hand, the highest and lowest mean Bursa index is seen on groups fed with Basil (59.21%) and Lemon grass (20.09%) respectively indicating better B- Cell production of basil fed chicken. Similarly, the highest mean spleen index is recorded on Basil group (40.78%), while the lowest record being thyme (10.43%) *(See Table 4).*

Table 4weight indices of lymphoid organs examined at 21 and 42 days

Feeding Trial	**Weight of immune organs**							
	DAY 21				DAY 42			
	Body Weight (Wt± SEM)	Thymus Weight (Wt± SEM)	Spleen Weight (Wt± SEM)	Bursa Wt (Wt± SEM)	Body Weight (Wt± SEM)	Thymus Weight (Wt± SEM)	Spleen Weight (Wt± SEM)	Bursa Wt (Wt± SEM)
Basil	440.3 ± 66.323978	.07133881 ± 0 .02994129	.08100191 ± 0.01442861	.24052211 ± 0.02974237	1211.7333 ± 140.34936	.37510038 ± 0.12889986	.12276819 ± 0.04426632	.17820969 ± 0.10507873
Lemon Grass	416.5 ± 15.276452	.08997376 ± 0.03681544	.05958559 ± 0.00825238	.22452855 ± 0.04314102	1389.6667 ± 244.9088	.52108425 ± 0.19935749	.37804999 ± 0.40163848	.16994231 ± 0.04633363
Mint	376.375 ± 11.820427	.07074481 ± 0.03416186	.19934843 ± 0.23636162	.24714916 ± 0.06226314	1075.47 ± 181.51749	.18837636 ± 0.03773754	.07547567 ± 0.00667259	.14249649 ± 0.04211896
Rose	397.7 ± 6.1392182	.14714561 ± 0 .04463289	.14960586 ± 0.04024678	.28768058 ± 0.16864753	1354.6 ± 175.6755	.58652751 ± 0.11407379	.09633501 ± 0.02378353	.24415132 ± 0.02209061
Thyme	428.55 ± 59.767633	.10995329 ± 0 .0236367	.08828302 ± 0.02535169	.34413277 ± 0.11621394	1229.9 ± 28.200532	.64755054 ± 0.17621655	.12407415 ± 0.03832067	.32689579 ± 0.07254777
Control	391.93333 ± 2.35017	.0945695 ± 0.01563172	.09543712 ± 0.03343791	.16052093 ± 0 .04233365	3530.6667 ± 4580.808	.27632196 ± 0.20568963	.06803426 ± 0.05165904	.11140041 ± 0.09143486
F-value	*1.38*	*2.59*	*0.78*	*1.55*	*0.75*	*4.31*	*1.85*	*3.56*
P-value	*0.2887*	*0.0732*	*0.5821*	*0.2373*	*0.6031*	*0.0204*	*0.1830*	*0.0368*

Weight indices of lymphoid organs (Thymus, Spleen and Bursa of fabricious) of chicken kept under six different feeding trials (Basil, Control, Lemon grass, Mint, Rosemary and Thyme) examined during 21st (Left) and 42nd days (Right).

Figure 1. Bar chart of the Mean index of lymphoid organ weigh index



Lymphoid organ weigh index (Thymus, Spleen and Bursa of fabricious) of chicken kept under six different feeding trials (Basil, Control, Lemon grass, Mint, Rosemary and Thyme) examined during 21st (Left) and 42nd (Right) days age.

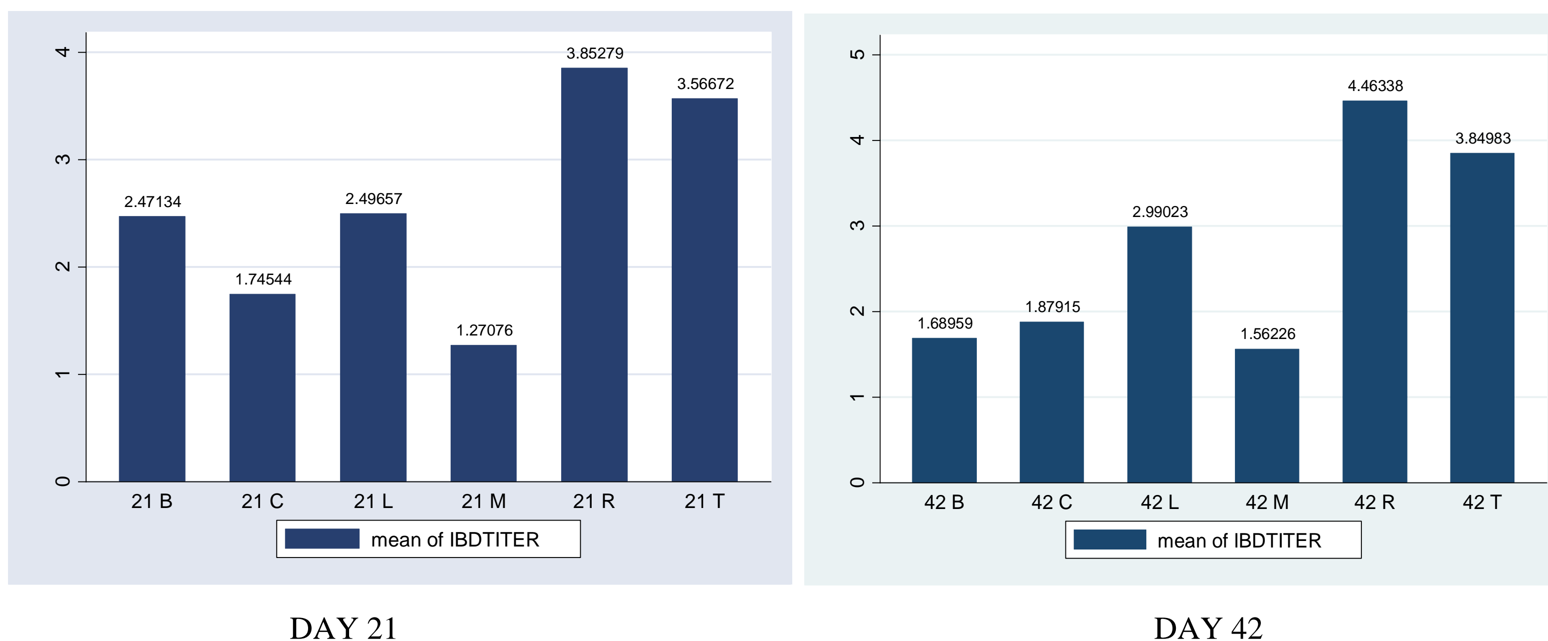
4.2.2 Antibody titer

Rosemary and Thyme have shown the highest mean Antibody titer ($\log_2 \pm$ SD) of (3.852789 ± 1.2895908) and (3.5667216 ± 1.1594758) respectively, against IBD on day 21 while the lowest titer was seen on group fed with Mint (1.2707589 ± 0.86111564). Similarly at the age of day 42, the mean antibody titers further increased in those same groups (Rosemary and Thyme), with their mean antibody titer being (4.4633804 ± 0.41798983) and (3.8498285+ 0.99124857) respectively followed by Lemon grass (2.9902251 ± 0.57872719). The lowest titer was recorded on groups fed on Mint (1.5622628 ± 0.34504147). Statistically significant difference was observed in the mean values of IBD antibody titers ($P < 0.05$).

On the other hand, Among a total of 42 serum samples examined from representative chickens of six different feeding trials who have previously taken Newcastle disease vaccine, 91.62% (41 of them) have shown protective level of immunity (> 1:8) against the vaccine, where only 2.38% (1 from 42) has shown immunity below protective level (<1:8) and which is from groups fed on Basil.

Groups of chicken fed with Thyme and Rosemary additives have shown the largest increase in antibody with their mean antibody titer in ($\log_2 \pm$ SD) being (5.6666±0.5773) and (5.6666±1.1547005) respectively whereas, groups of chicken fed Basil, has shown the least antibody titer of (4.3333±2.0816). Similar scenario of changes in antibody titer was observed on the first phase of study (day 21), except that Mint is the group where the lowest antibody titer of (4.666± 0 57735) was seen while the largest being Thyme and Rosemary (6.3333 ±0.57735)

Figure 2 Bar chart of Mean antibody titer against IBD vaccine



of chicken kept under six different feeding trials examined during 21st (left) and 42nd (Right) days' age. B= Basil= Control, L= Lemon grass, M= Mint, R= Rosemary, T= Thyme, (21 and 42) = Ages of chicken when examined.

5. DISCUSSION

Supplementation of broilers with PFA generally, had favorable effects in that they significantly reduced pathogenic bacteria load and enhanced the normal flora to proliferate.

5.1 Cecal bacterial load

As of the finding of this study, the lowest *E. coli* counts were seen on groups fed at Rosemary. Lemon grass, Basil and Mint have also lower count of the cecal *E. coli* load next to Rosemary. This could be due to the fact that these herbs contain volume of essential oils (active substances) which have antimicrobial effect inhibiting the growth and proliferation of pathogenic bacteria.

Similar finding was reported by (Weckesser *et al.*, 2007) where Rosemary extract has significantly reduced pathogenic bacteria load. A good to moderate antimicrobial activity of Rosemary essential oil has also been reported by several other authors (Gachkar *et al.,* 2007).

On the other hand, Rosemary and Lemon grass has significantly increased the gut *Lactobacilli* count ($P < 0.05$) followed by Mint grass and Basil. The statistical analysis of this data, in general indicated that those herbs which have reduced the gut *E. coli* load increased the cecal *lactobacilli* population and this might attribute to the property of *Lactobacillus spp*. in excluding pathogens adhesion to mucosal wall due to their rapid colonization, acidification, and proliferation in gastrointestinal tract (Aoudia *et al.*, 2016).

The highest cecal *Enterococcal* count count rccorded during 21st day was by Lemon grass followed by Thyme. The mean logarithmic bacterial count was relatively similar on all test herbs though the highest count was recorded for Thyme followed by Mint and Lemon grass compared to the lowest count on Control group. The statistical analysis of the data in the two phases of experiment indicated that almost all of the test herbs have improved the gut load of beneficial bacteria where, Thyme, lemongrass and mint holding the top

ranks, respectively. This finding is in agreement with *(Ahsan et al.,* 2018) who supplemented broiler diets with different levels of phytogenic feed additives and tested the dynamics of growth performance, cecal micro biota, and intestinal morphometry.

5.2 Effect on immunity

5.2.1 Lymphoid organ weight

Various lymphoid organs such as: bursa of fabricious, thymus and spleen from chicken of six experimental feeding trials were also weighed twice during the experimental period (day 21 and 42). Accordingly, Lemon Grass, Rosemary and Thyme have shown the highest percentage in mean thymus index than the other groups which could reflect increased production of plasma cells. On the other hand, the significant increase in mean thymus index post-vaccination could be due to increases in the number of activated macrophages that could prime the T cells to proliferate and produce cytokines that enhance antibody production. Similar finding was reported by (Aly *et al*., 2019)who experimentally fed broiler chicken with Biostrong 510, and examined lymphoid organs weight to body weight ratio where, significant increase in the ratio of thymus weight to total body weight was observed. Similarly, the highest mean Bursa index is seen on groups fed with Thyme indicating better B- Cell production of Thyme fed chicken. This finding was in agreement with (Alipour *et al*., 2015), who supplemented broiler chicken with dietary thyme-oil extract (TOE) and assessed the immune functions after subjecting them with 0.5 ml of 10% SRBCs where broilers fed with 200 parts per million (ppm) of Thyme-oil extract had relatively much heavier weights of bursa than those fed other dietary treatments at day 42 of their age.

5.2.1 Antibody titer

The increase in antibody titer on groups fed with thyme and rosemary could be due to the volume of essential oil in these plants Thymol and carvacrol from (Thyme) and Cineol from (Rosemary), which are considered as appetizers besides their antimicrobial activity against intestinal bacteria. (Jameel *et al*., 2014) also confirmed similar result that supplementing broiler diets with 1% thyme herbal feed additive significantly improved the immune response of broilers. Similar result was also reported by (Genena *et al.,* 2008) who tested The rosemary extracts with regard to antioxidant (DPPH radical scavenging and total phenolic content), antiviral, antibacterial and antifungal (*Candida albicans*) activities.

6. CONCLUSION AND RECOMMENDATIONS

Result of the present study indicated that supplementation of broilers with PFA generally, had favorable effects in that they significantly reduced pathogenic bacteria load and enhanced the normal flora to proliferate. These results suggest that herbs produced in Ethiopia could be used as phytogenic feed additives to minimize risks of infections with pathogenic bacteria and vaccine failures. However, detail scientific investigation on their medicinal use and their combined formulation as feed additive is lacking in our country context beyond their traditional use only.

From the above conclusion the following recommendations are forwarded.

- Further detail studies which combine both *in-vitro* and *in-vivo* investigation should be conducted for better discovery of their medicinal and any other effect beyond solely reporting their traditional use.
- Their effects in different possible combinations should be well studied so that, herb that show only mild to moderate effect, may perform great when combined with other herbs.
- Phytogenics is not a well explored area of science in our country and therefore still much has to be done to discover their novel effects beyond yet discovered so far.

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8. ANNEXES

Annex 1 General design of the Study

Feeding trials																	
Lemon grass			Mint			Thyme			Basil			Rosemary			Control		
Replicates			Replicates			Replicates			Replicates			Replicates			Replicates		
1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks	20 chicks

Annex 2 Activity plan of the research

Experimental activities		Experimental Period (Days)
Period of acclimatization		1-5
Vaccination	NCD	2 (HB 1), 12 (Lasota), 24 (Lasota)
	IBD	7, 19
Blood collection for hematology and immunology		1, 21, 42
Cloacal Swab/ caecal sample collection		7, 21, 42

Annex 3 Bacterial isolation and identification routines

Experimental activity	Tasks and details	
	Media preparation	Nutrient agar/broth, MacConkey, XLD, EMB, MRS, SIM, TSI, MR-VP
Bacterial isolation and identification	Biochemical identification	Gram staining, catalase test, IMV_iC Test, motility, sugar fermentation, H2S production, Gas production
	Bacteriological quantitative assay test	*E. Coli* Count, *Lactobacillus* Count, *Enterococc*i Count

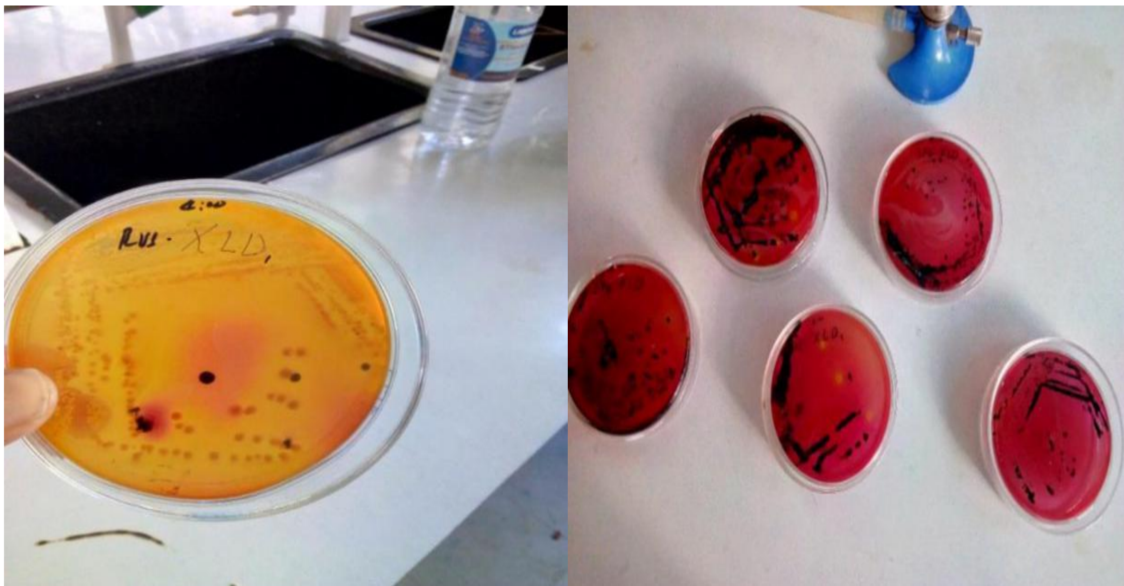
Annex 4 Lymphoid organ weights recording format

Treatment Group	Code	weight of immune organs			
		weight of the chick	thymus	spleen	bursa of fabricious
Thyme	T1				
	T2				
	T3				
Rose	R1				
	R2				
	R3				
Basil	B1				
	B2				
	B3				
Mint	M1				
	M2				
	M3				
Lemon Grass	L1				
Control	L2				
	L3				
	C1				
	C2				
	C3				

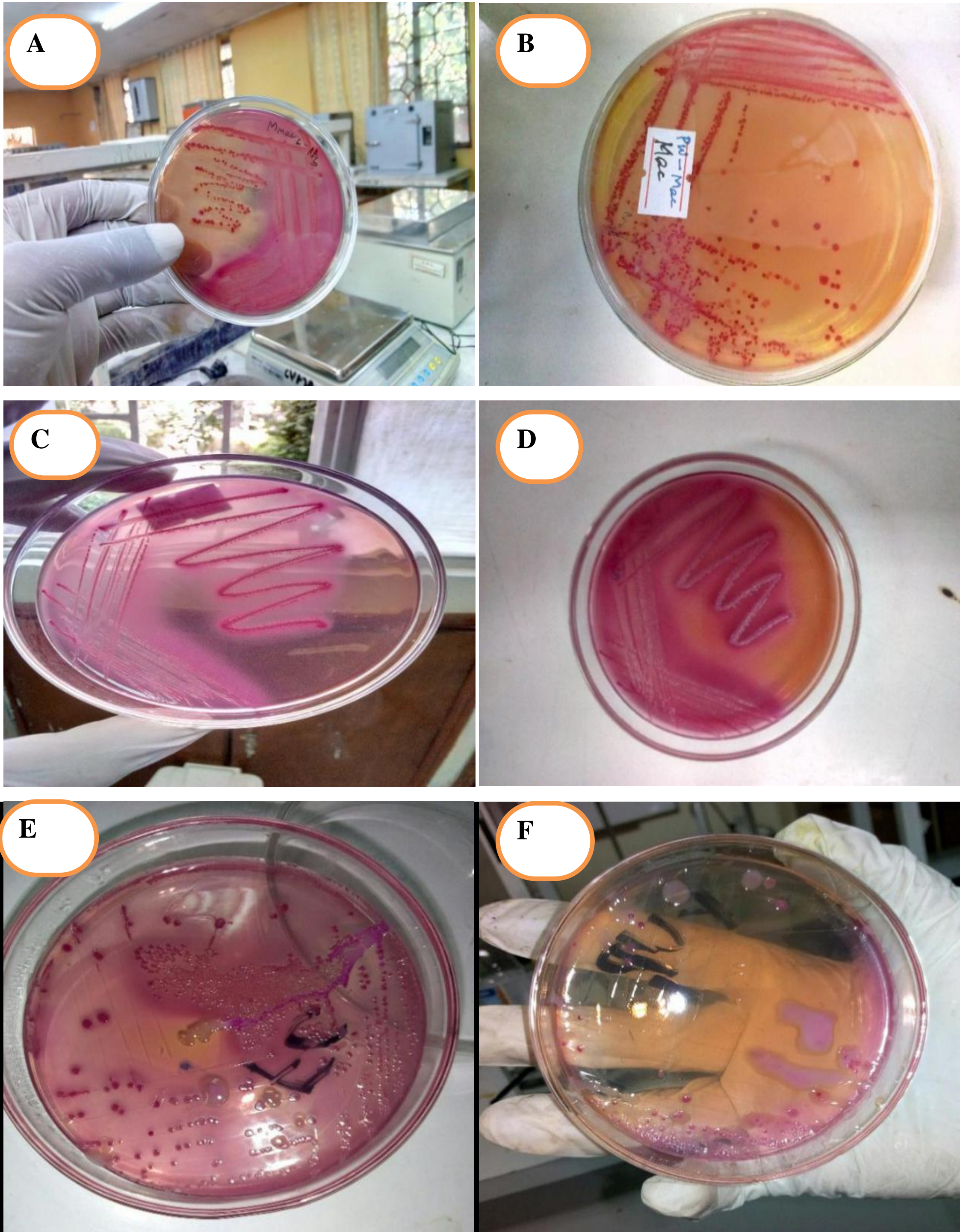
Annex 5 Different pictures indicating bacterial isolation and identification

A/ Bacterial isolation and identification

Pink colonies with black center indicating *Salmonella* species



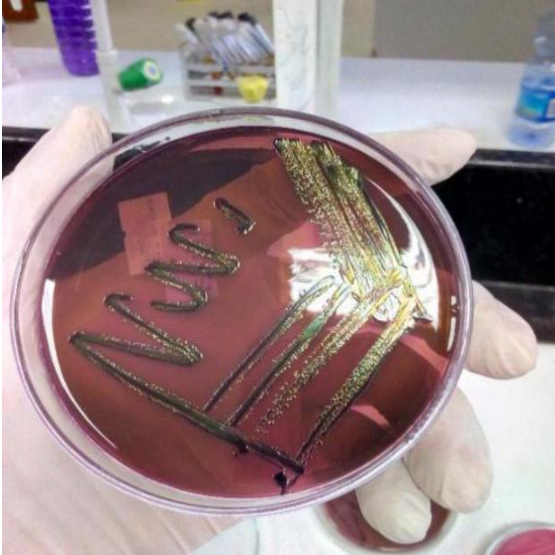
Yellow colonies (E.coli, proteus, klebsiella) Red colonies with black center (*Salmonella*)



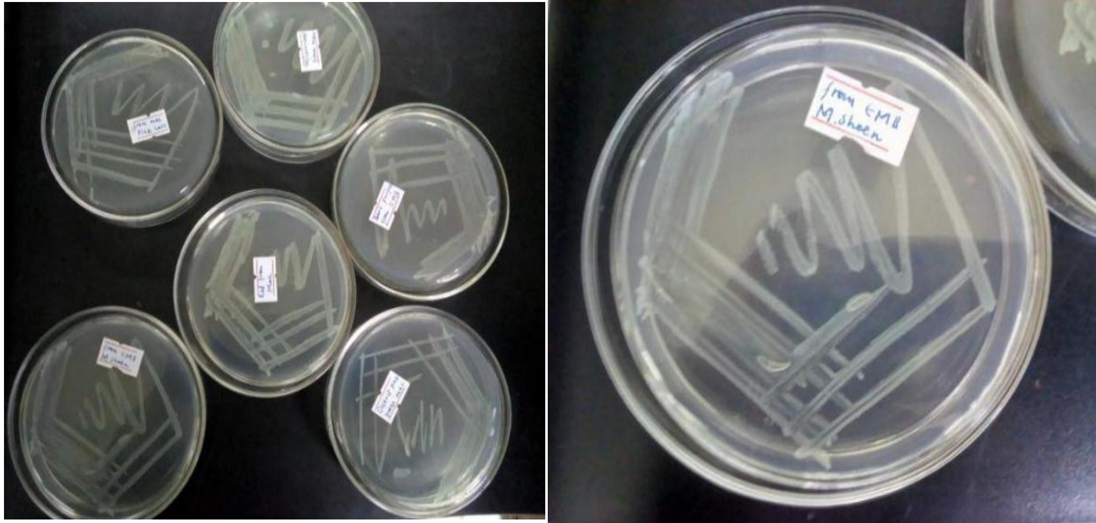
Non-Lactose fermenting, Colorless/ yellowish colonies (top row A and B *Salmonella*, proteus)

Lactose fermenting Pink colonies with a halo of bile precipitate (mid-row C and D, *E. coli*),

Lactose fermenting Pink mucoid colonies (bottom row E and F, Klebsiella)



Greenish metallic sheen colonies sub cultured from pink halo of MacConkey to EMB Agar (E. coli)

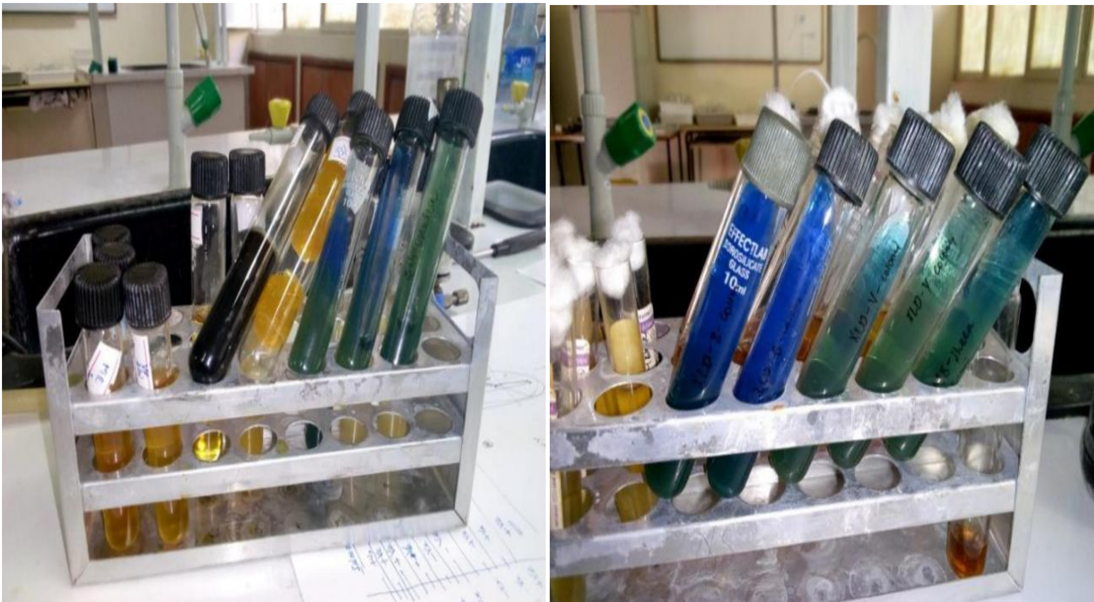


Pure colonies subcultured on nutrient agar for further biochemical characterization

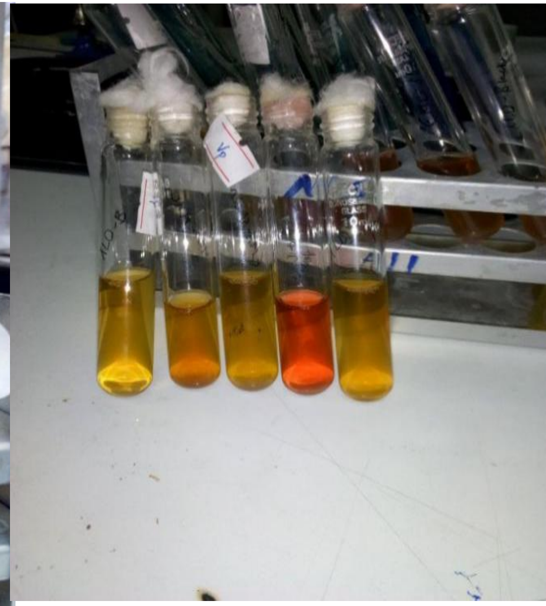
Anaerobic culture of *lactobacilli* using anaerobic jar and lighted candle inside

Gram staining and Biochemical tests

Biochemical tests



Mot
Mot

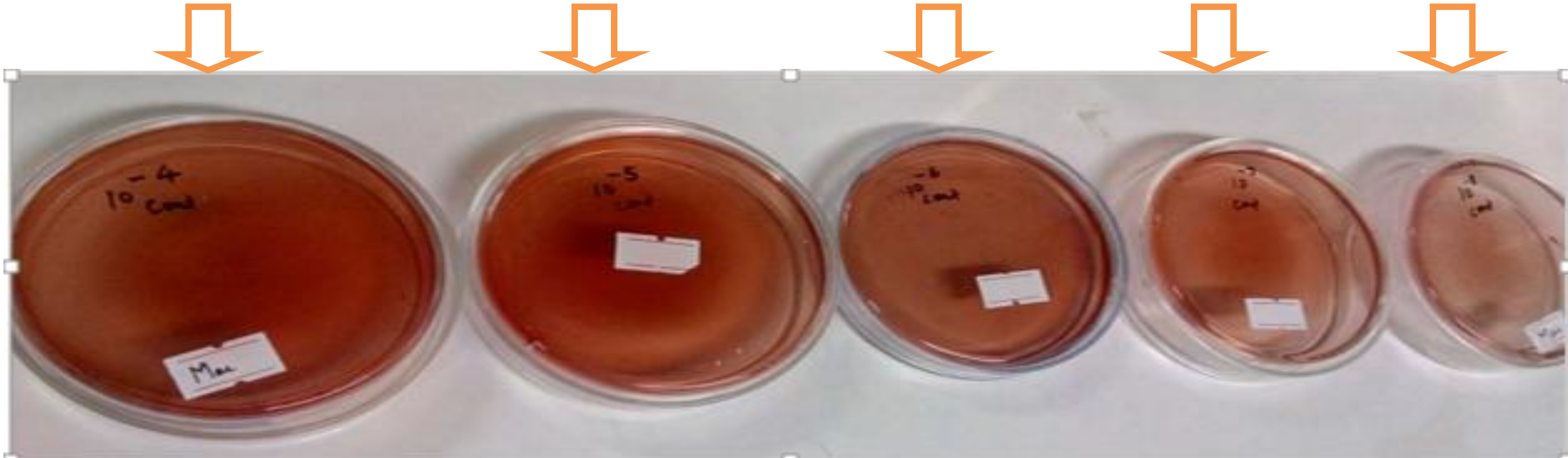


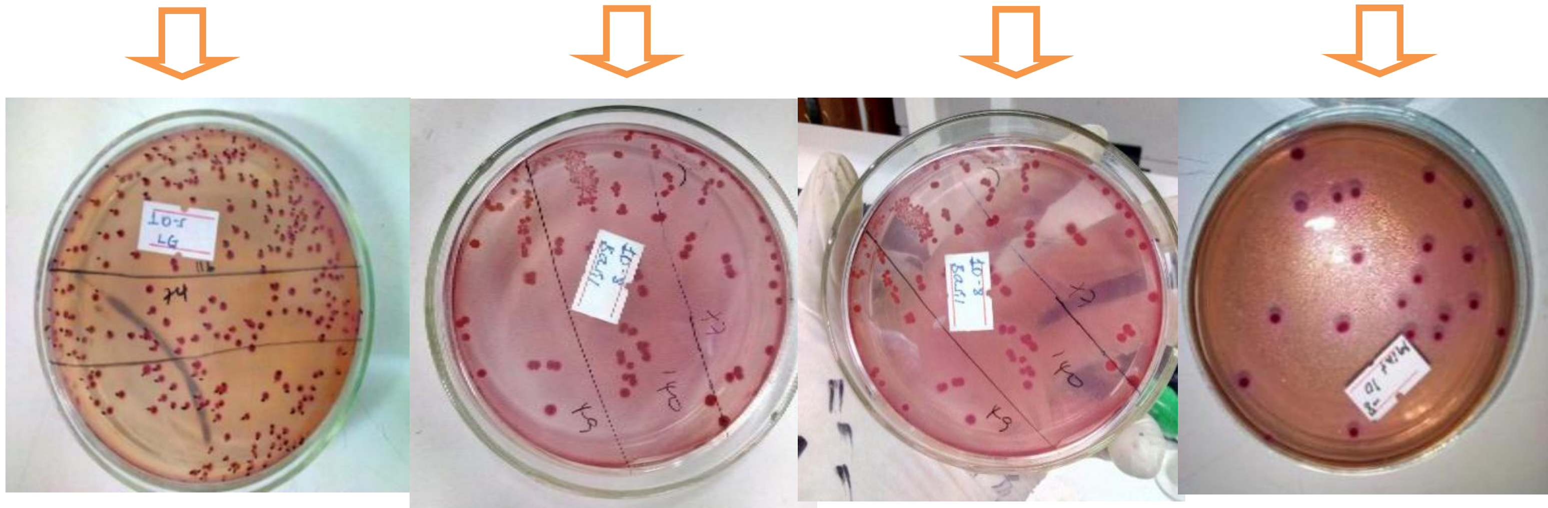
B/ Bacterial enumeration

Serial dilution among different treatments and bacterial enumeration

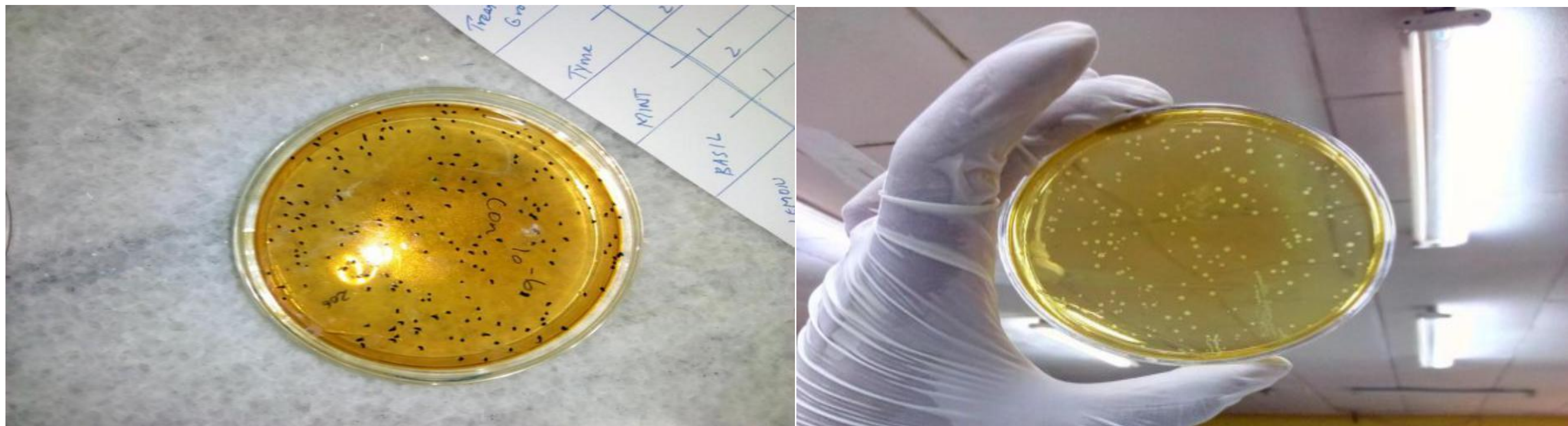
Serial dilutions (control group)

Serial dilutions (treatment groups)

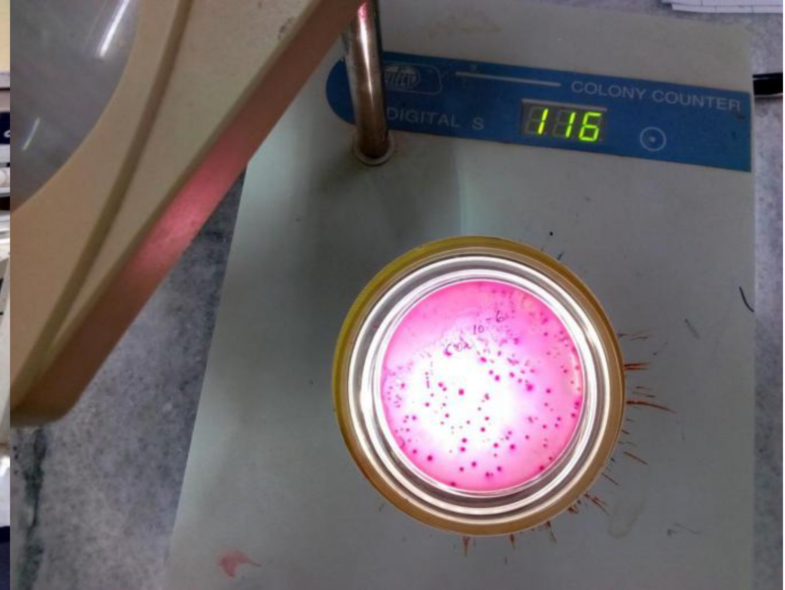




10^{-5} (Too numerous to count) 10^{-6} dilution 10^{-7} dilution 10^{-8} dilution *(Too few to count)*



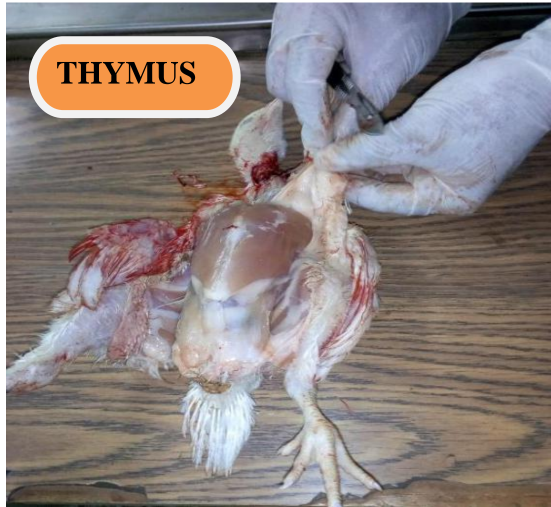
MRS Plates having countable colonies (30 to 300) chosen among dilutions plated

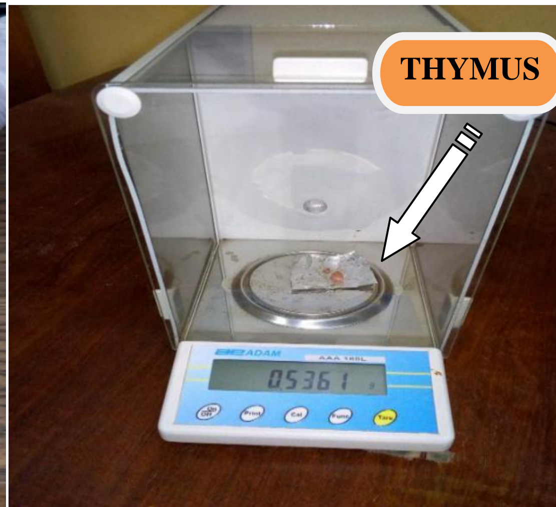


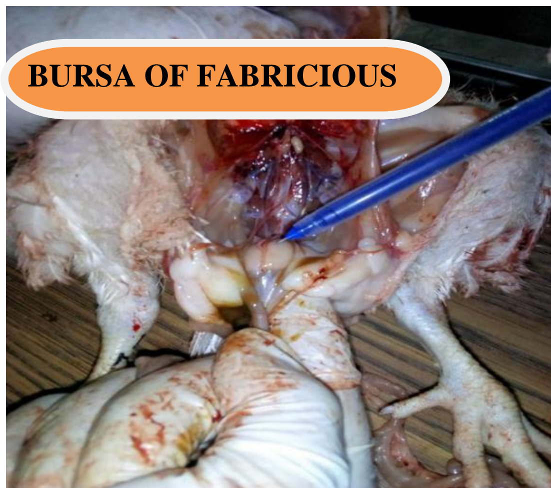
Enumeration of bacterial colonies using colony counter

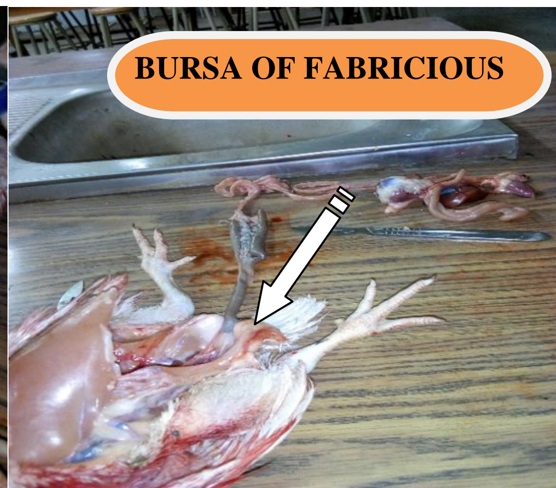
116 colony forming unit per ml

C/ Lymphoid organ collection and weighing during post mortem









Annex 6 Haemagglutination inhibition protocol

(Sano and Ogawa, 2014)

Materials

- *Thawed serum samples in racks*
- *V-bottom micro well plates*
- *PBS*
- *1 percent washed red blood cells*
- *V-bottom reagent trough*
- *25 µL pipettes and tips*
- *Micro well plate recording sheet.*
- *Newcastle disease virus antigen diluted to 4 HA units per 25 µL*
- *Standard positive and negative serum*

Method

1. Fill in recording sheets to record how samples will be dispensed into micro well plates.
2. Calculate the number of plates required and number each plate.
3. Dispense 25 µL of PBS into each well of the plates.
4. Shake each serum sample and dispense 25 µL into the first well and the last (control) well of a row of a micro well plate.
5. Use a multichannel pipette to make two-fold serial dilutions along the row until the second last well from the end. The last well is the serum control. Do not dilute this well.
6. Add 25 µL of the 4HA dilution of antigen to each well excluding the control in last column.
7. Gently tap the sides of the micro well plates to mix the reagents. Cover plates with a lid. Allow to stand for 30 minutes at room temperature.
8. Add 25 µL of 1 percent washed red blood cells to each well including the control wells in the last column.
9. Gently tap the sides of the micro well plates to mix the reagents. Cover the plates with a lid. Allow to stand at room temperature for 45 minutes.
10. Read the settling patterns for each serum sample. Read the control serum well first then read the patterns in the other wells.

11. Record the pattern observed in each well on a micro well plate recording sheet. Determine the endpoint. This is the point where there is complete inhibition of haemagglutination.

12. Record the antibody level for each serum sample. This is expressed as a log base 2. For convenience, the titer is often recorded as just the log index. For example a titer of 2^6 would be recorded as 6.

Interpretation of results

- In the wells where antibodies are present there will be haemagglutination inhibition. The red blood cells will settle as a button.
- In the wells where antibodies are absent, the red blood cells will agglutinate.
- The end point of the titration is the well that shows complete haemagglutination inhibition. Sometimes it is not easy to determine. Look at the size of the button as an indication of the degree of haemagglutination inhibition. Use the control well as a point of comparison. Be consistent in determining the endpoint.

Annex 7 Indirect ELISA protocol

(Al Suleiman *et al.,* 2020)

Procedure

An indirect ELISA is one where the primary antigen-specific antibody is recognized by a secondary conjugated antibody. The following protocol is an example of an indirect ELISA method, where the serum samples of of influenza A virus (IAV)-infected mice are tested for the presence of IAV-specific IgG antibody. One strength of this example is that different secondary antibodies can be used that recognize all antibody isotypes or specific isotypes (e.g., IgG).

Coating antigen to the micro plate

1. Coat the wells of a 96-well ELISA plate with purified antigen by pipetting 50 µL of purified antigen into each well of the plate.
2. Cover the plate with an adhesive cover and incubate it overnight at 4°C to allow the antigen to bind to the plate.
3. Upon completion, remove the coating solution by flicking the plate over a sink.

Blocking

4. Block the remaining protein-binding sites in the coated wells by adding 200 µL blocking buffer, 5% donkey serum in 1X PBS is used here, per well. Alternative blocking reagents include 5% non-fat dry milk or BSA in PBS or normal serum from an animal in which the secondary antibody was generated.
5. Incubate for at least 2 hours at room temperature or overnight at 4°C.
6. Following the incubation, remove the blocking buffer by flicking the plate and then wash plate with PBS containing 1% Tween-20.

Incubation with the primary antibody

7. Prepare a serial dilution of the serum sample, which contains the primary antibody, to obtain a dilution range of 1 to 204,800, using 1X PBS. To do this, first dilute the serum 1:12.5 and then perform a 4X dilution (dilution range - 1:12.5 to 1:204,800).

8. Add 100 µL of the serially-diluted serum samples to the wells.
9. Cover plate with adhesive cover and incubate at room temperature for 1-2 h.
10. Following the incubation, flick the plate over a sink and wash plate with PBS containing 1% Tween-20.

    <u>*Incubation with the secondary antibody*</u>

11. Add 100 µL of an enzyme-conjugated secondary antibody, horseradish peroxidase, HRP-conjugated donkey anti-mouse secondary in this experiment, to each well.
12. Incubate the plate for 1 hour at room temperature.
13. Following the incubation, flick the plate over a sink and then wash plate with PBS containing 1% Tween-20.

    <u>*Detection*</u>

14. Add 100 µL of the indicator substrate (3, 3', 5, 5'-tetramethylbenzidine (TMB) at a concentration of 1 mg/ml to each well.
15. Incubate the plate with the substrate for 5-10 min at room temperature.
16. After 10 min, stop the enzymatic reaction by adding 100 µL 2N Sulfuric acid (H_2SO_4).

    Within 30 min of adding the stop solution, read the plate using a micro plate reader at 405 nm to determine the absorbance of the wells.