Ministry of Higher Education

and Scientific Research

University of Kerbala

College of Science

Department of Biology

Effect of *Bacillus subtilis* on microbial community , growth performance, and some haemato - immunological parameters of common carp (*Cyprinus carpio*)

A Thesis

Submitted to the Council of College of Science at University of Kerbala in Partial Fulfillment of the Requirements for the Master Degree in Biology

By

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2021AD **1442AH**

"يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا
مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ
دَرَجَاتٍ وَاللَّهُ بِمَا
تَعْمَلُونَ خَبِيرٌ"

صدق الله العلي العظيم
سورة المجادلة { 11 }

Supervisors Certification

We certify that this thesis was prepared under our supervision at the College of Science, University of Kerbala, as a partial fulfillment of the requirement for the Degree of Master of Science in Biology.

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DECISION OF THE EXAMINING

We are the examining committee, certify that we have read this thesis entitled " **Effect of *Bacillus subtilis* on microbial community , growth performance, and some haemato - immunological parameters of common carp (*Cyprinus carpio*)** " and examined the student **" Doaa Ali Beiwi AL -Ghanmi** " in its contents, and that according to our opinion, it is accepted as a study in partial fulfillment of the requirements the degree of **Master** of Science in Biology with ( ) estimation.

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Dedication:

To the prophet Mohammed and Ahl al –Bayt (peace be upon them)

To Imam Al Mahdi Al Montazer (peace be upon him and Hurry Allah reappearance)

To my angels in life... To the meaning of love and compassion who taught me the meaning of life and their existence is the reason for my success....

….My parents …

Doaa Ali AL -Ghanmi

Acknowledgment:

Praise be to Allah and peace and prayer upon to the prophet Mohammed and his progeny

I want to express my great thanks to Almighty Allah for his uncountable gifts and for helping me to present this thesis

I express my deepest gratitude to my supervisor, **Dr. Ali A. Al-Hisnawi** for his great support and his guidance during study.

I would like to express my gratitude to staff members of the department of Biology/ College of Science/ University of Kerbala, for offering the scientific support, education and facilities, special thanks to the **Dr. Jasem Hanoon Hashim** and **Dr. Wafa Sadiq Al-Wazni**.

I would like to express my gratitude to my family for supporting and helping me.

I would also like to thank everyone and my dear friends **Methaq Rashid Sharqi and Naba Kazim Mohammed** for helping me.

God Bless all of you

Abstract

The current study was carry out for aperiod of six weeks from 24 February to Aprile/ 2019 and conducted to investigate the influence of dietary supplementation of *Bacillus subtilis* on intestinal microbial community, growth performance, haematological parameters, serum biochemistry parameters and the levels of cytokines including transforming growth factor beta (TGF β) and Interleukin1beta (IL1 β) of common carp. A total of 70 fish (77.2 ± 0.86 g) were collected from AL-hafiz farm a local commercial at Karbala city and randomly distributed into (14) groups in plastic tanks, each group contain five fish. Seven tanks were allocated for the probiot group and the other seven tanks were allocated for the control group. Fish were fed at 1.5-2% of body weight twice daily.

The probiotic was isolated from local soils and using polymerase chain reaction (PCR) technique for the amplification of Ribosomal ribonucleic acid (16S rRNA). Fish were fed a diet containing *B. subtilis* at dose of ~10^7 CFU g^{-1} control diet for 6 weeks. Total viable count (TVC) bacterial populations and *subtlilus* were enumerated using tryptone soy agar (TSA) and de Man, Rogosa and Sharpe agar (MRS),respectively.

The results of microbiology revealed that the resident total viable count (TVC) and lactic acid bacteria (LAB) microbial community significantly decreased in the probiotic fed fish group compared to the control group, while the microbial community of TVC in the digesta samples was significantly increased in the probiotic fed fish compared to the control group. The bacterial diagnosis for microbial community of

common carp revealed that the communities grouped into two distinct phyla, Proteobacteria and Firmicutes.

The results of growth performance profiles were not influenced by probiotic diet. Compared to the control group, the carp fed probiotic supplemented diet displayed a significant elevation in mean corpuscular haemoglobin concentration (MCHC), ($P < 0.05$). The other haematology parameters were not significantly affected. The blood serum profiles i.e. blood urea, cholesterol and random blood sugar, were not affected by the experimental diet.

However, serum creatinine was significantly higher in the probiotic group compared to the control group. The experimental carp displayed elevation level of IL1β compared to the control group (P=0.004). On the other hand, the level of TGFβ was lower in the probiotic treated fish but no significant differences were observed (P=0.05).

The results of the present study, demonstrate potential role for *B. subtilis* to modulate intestinal microbiota and the health status of common carp.

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List of Abbreviations

AD C	Anterior digesta control
ALT	Alainin amino transferase
AMC	Anterior mucosa control
ANOVA	Analysis of variance
AMPs	Anti-microbial peptides
ACC	Aerobic colony count
B	Biomass
BCR	B cell receptor
BUN	Blood urea nitrogen
CFU	Colony forming unit
CCVD	Channel catfish virus disease
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immuno sorbent assay
EMB A	Eosin methylene blue agar
FAO	Food and agriculture organization of the united nations
FCR	Feed conversion ratio
FI	Feed intake
FISH	Fluorescent in situ hybridization
FM	Fish meal
FW	Final weight
GIT	Gastrointestinal tract
GRAS	Generally regarded as safe
Hb	Haemoglobin
HCT	Hematocrit
IELs	Intra epithelial leucocytes
IH N	infectious haematopoietic necrosis
IPN	Infectious pancreatic necrosis
IECs	Intestinal epithelial cells
IL	Interleukin
IN	Initial weight
IL1β	Interleukin 1 beta
Kg	Kilogram
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration

MCT	Microcentrifuge tubes
MCV	Mean corpuscular volume
Mg	Miligram
MHC	Major histocompatibility complex
Min	Minute
Ml	Mililiter
MRS	De -Man, rogosa and sharp
MW	Molecular weight
NB	Nutrient broth
NCC	Non-specific cytotoxic cells
NK	Natural killer
OD	Optical density
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P.C.V	Percentage packed cell
PCNA	Programmed cell necrosis apoptosis
PD	Posterior digesta
PDC	Posterior digesta control
PH	Potential of hydrogen
PMC	Posterior mucosa control
RBC	Red blood cells
RBS	Random blood sugar
RNA	Ribonucleic acid
Rrna	Ribosomal ribonucleic acid
SGR	Specific growth rate
TAE	Tris-acetate- EDTA
TCR	T lymphocyte cell receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TSA	Tryptone soy agar
TVC	Total viable counts
WBC	White blood cells
WG	Weight gain
WHO	World health organization
WSSV	White spot syndrome virus

Chapter one
Introduction and Literature review

1-1 Introduction

In the years aquaculture industry is demand as a rapidly growing sector and a major contributor in providing high-quality animal protein. Common carp (*Cyprinus carpio)* is the world's third most commonly grown and commercially significant species of freshwater fish (FAO 2013). It is also considered one of the most important freshwater aquaculture species grown in Iraq. However, common carp are easily infected with pathogenic microbes associated with high density culture conditions which always lead to the decrease of immune level (Wang, 2017). Two years ago, the aquaculture industry in Iraq in particular intensively faced deterioration of water quality which resulted in the outbreak of fish diseases. Antibiotics to control disease outbreaks are no longer suggested due to several negative effects on the environment, followed by the growth of development of antibiotic-resistant microorganisms and adverse impacts on fish health which leads to food safety problems for human health (Mo *et al*., 2017). Additionally, antibiotics in aquaculture products may be harmful to human health by inhibiting or killing the normal microbiota of the digestive tract (Aly *et al.,* 2008). This situation resulted in the search for a promising alternative approach for antibiotics in aquaculture to control potential pathogens (Aly *et al*., 2008; Dawood *et al*., 2018). Thus, the use of probiotics is an Eco-friendly approach that has been found to enhance the physiology, growth efficiency, and immune reactions of aquaculture-related species. Gram positive spore forming *Bacillus* spp. and lactic acid bacteria have been commonly applied as probiotics in fish and shellfish farming (Wang *et al*., 2008).

For less than 50 years, the geneus of *Bacillus* has been used as a probiotic. The species which are used more include *Bacillus subtilis* and *Bacillus licheniformis.* These bacteria when used as probiotic in form of a feeding supplement will lead to the stimulation of the immune system ,and have antimicrobial activity and prevent competition (Cutting, 2011). *B. subtilis* grow effectively with low-cost sources of carbon and nitrogen because their enzymes are very effective in breaking down a wide range of animal and vegetable proteins, carbohydrates and lipids into their constituent units (Olmos and Paniagua-Michel, 2014).

Bacillus spp. have several benefits due to their spores resistance to heat and high acidity with a long lasting shelf life for producing mass scale commercial feed (Elshaghabee *et al*., 2017). Although probiotic applications have been widely used in fish and shellfish aquaculture, no information is obtainable concerning probiotic supplementation into common carp in Iraq.

The aim of study

The present study was carried out to investigate the influence of dietary supplementation of probiotic bacteria (*Bacillus subtilis*) on health status of (common carp) fish.

The objectives were performed by the following:

- Characterization of the gasterointestinal tract (GIT) microbiota including lactic acid bacteria (LAB) of common carp as a result of administration of probiotic *Bacillus subtilis* as feed additive to common carp.

- Investigation of the effects of *Bacillus subtilis* on the innate immune system (including hematology parameters and some pro- and anti-inflammatory cytokines).
- Determination of the effects of *Bacillus subtilis* on functions of kidney and liver by check some biochemistry parameters.
- Evaluation of growth performance of common carp by the efficacy of probiotics in diets.

1-2 Literature Review

1-2 -1Aquaculture

Over the past few decades, the aquaculture industry has evolved quickly and it has played a major part in contributing to future protein requirements for humans (Abdel-Tawwab & Ahmad, 2009; Godfray *et al*,. 2010).

Global aquaculture production within the Asian region as a whole was recorded at 73.8 million tons in 2014, producing more farmed fish than wild fish since 2008, with total production reaching 44.1% in 2014, up from 42.1% in 2012. One of Iraq's major animal sector is the fish sector, which accounts for about 3% of total financial yields and produces up to 113 million tons of fish meat annually (Al-Mahmood, 2017).

Common carp (*Cyprinus carpio*) is part of the Cypriniformes order and the Cyprinidae family, which is known to be the largest freshwater fish family. It usually lives in freshwater environments, especially wetlands, lakes and rivers, and also rarely lives in brackish-water environments (Barus *et al*,. 2001). It is widely distributed in nearly every country in the world, but is common in Asia and some European countries (Weber and Brown, 2011; Kloskowski ,2011; Parkos and Wahl 2014). Common carp is the world's third most widely introduced species. It is considered a potential candidate for commercial aquaculture in Asia and some European countries, because it has a very high capacity for environmental and food adaptation (Soltani *et al*., 2010; Manjappa *et al*. 2011; Rahman 2016).

More than 80% of total fish production in some European countries comes from common carp (Woynarovich *et al.* 2010; Anton-Pardo *et al*., 2014).

It is commonly stated that common carp is often referred to as an ' ecological engineer ' because it can alter the ecological character of water systems (Shin-ichiro, *et al*., 2009; Bajer and Sorensen, 2015; Rahman, 2015).It is noteworthy that common carp is the world's third most commonly grown and commercially significant species of freshwater fish (FAO, 2013). It is also considered to be the most common species of fish grown in Iraq. It has many features that make it appropriate for the aquaculture sector in Iraq, including rapid increase in weight during short time as well as resistance to many pathogens in the aquatic environment of Iraq .The quality and quantity of aquaculture methods have been improved by such factors including an increase of investors who are interested in this sector and new techniques of fish breeding. The increase was accompanied by the creation of new fish ponds using new breeding techniques such as cages and breeding equipment as well as an increase in the number of fish hatcheries in Iraq supplying fish fingerlings ((Al-Mahmood *et al* ., 2017).

The rise of commercial aquaculture and intensive fish processing around the world has unfortunately increased the risk of infectious disease. Bacterial disease for example has been confirmed to be a major cause of death in hatcheries of fish (Grisez and Ollevier, 1995).

On the other hand, diseases have been considered to become the main constraint of aquaculture production globally with the rapid development of commercial aquaculture farming (Arthur& Subasinghe, 2002).

Once the ecosystem has weakened, most pathogens are opportunistic to infect the developing fish. In the meantime, bacterial diseases also targeted fish grown followed by parasites, viruses and fungi. Among the

bacterial species, the disease of vibriosis and aeromonad pose a threat to most marine or freshwater farms. (Toranzo *et al.,* 1995).

In decades, antibiotics has been used as a traditional strategy for the management of fish diseases and also for the improvement of feed conversion growth and efficiency. Nevertheless, the risk is well known for the development and spread of antimicrobial-resistant pathogens (Cabello and Sorum, 2006). Their use to control disease outbreaks is no longer suggested due to the adverse impacts of chemicals and antibiotics on the environment, followed by the growth of mutagenic microbial strains and adverse impacts on fish health (Cabello, 2006).

In many countries antibiotics administration have been avoided for the production of animals to be used as human food. The consumer market demands high-quality, high-security and healthy food that is free of chemical residues. Research on the feed's functional ingredients, such as probiotics, were thus strengthened. These constitute a viable and safe alternative to chemicals used in animal production to prevent diseases .Aquaculture probiotics have been used as growth promoters, immune system modulators, growth pathogen inhibitors, and to improve stress tolerance (Verschuere *et al*., 2000; Wang *et al*., 2008).

The use of eco-friendly feed additives, such as microbial supplements, to enhance the physiology, growth efficiency, and immune reactions of aquaculture-related species has therefore received much more attention in the years (Dawood, 2016; Song, 2014) . These are a feasible and secure solution to chemicals used in animal production for disease prevention.

Considerable attention has been devoted to probiotics or microbial supplements which are the best option to be used instead of antibiotics (Deeseenthum *et al*,. 2007). Probiotics are known as supplemental

microorganisms such as bacteria, fungi and yeasts which increase the health of the host through balancing the microbial flora of the gastrointestinal tract (Vivas *et al*,. 2004, Agouz and Anwer, 2011).The species which are used more include *Bacillus subtilis* and *Bacillus licheniformis.* These bacteria when used as probiotics in form of the feeding supplement, will lead to the stimulation of the immune system and have the antimicrobial activity and prevent competition (Wang *et al*., 2008; Lakshmanan and Soundarapandian, 2008; Cuting, 2011; Farzanfar *et al.,* 2009). The use of probiotics in aquaculture is thus anticipated to be an excellent strategy for the prevention of infectious microbial diseases and to replace antibiotics and chemotherapeutics (Balcázar *et al*,. 2006).

1-2-2 Intestinal microbiota of fish

The microbiome, an animal-associated collection of microorganisms, is important for the optimal growth and survival of the host species (Human, 2012 ; Ray *et al.,* 2012). The digestive tract microbiota, in particular, plays an integral role in food digestion, energy supply, vitamin development and innate immunity formation (Sampson and Mazmanian, 2015). Microbial community found within the GI tract of both terrestrial and aquatic animals can be divided into two main categories; either allochthonous (transient) or autochthonous (adherent) microbial communities (Nayak, 2010; Ringø *et al.,* 2006) these microbial populations consist mainly of bacteria but also some yeasts. Natural microbes populations can be influenced by various exogenous and endogenous factors including feed, chemicals, environmental conditions, pressure and pesticides, all of which can significantly change the gasterointestinal tract (GIT) balance. Since some microbial populations support the host

organism, it is vital to understand the interactions between the microbial populations of the GI tract and the host organism. (Nayak, 2010).

Aerobic, facultative anaerobic and obligate anaerobic bacteria, unlike for terrestrial vertebrates, are the main colonizers in the fish GI tract (Llewellyn *et al.*, 2014). Studies have shown that the fish gut is estimated to contain 10^7 to 10^{11} CFU in one gram of intestinal content (Nayak, 2010). Research has shown that bacterial members of Proteobacteria, Fusobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Clostridia, Bacilli and Verrucomicrobia are the most common colonizers in fish tract (Carda-Di_eguez *et al.*, 2014).

It is well documented that GI tract microbiota are affected by a number of fish parameters including host factors (e.g. genetics, sex, weight, age, immunity, and bowel motility) (Li *et al.*, 2016) conditions of the environment (e.g. heat, food, medicine and antibiotics) (Ringø *et al.*, 2016; Dehler *et al*., 2017), and microbial factors (e.g.adhesion ability, enzymes and metabolism) (Prakash *et al.*, 2011).

Therefore, it is reasonable to postulate that in the gut bacterial flora, lactobacillus and bacillus are considered important and more dominant. Studies showed that species of *Bacillus* cause changes within the fish gut preventing the invasion of pathogenic organisms in the inner bacterial microflora composition and help to maximize the use of feed (Krishnan ,2014).

1-2-3 The importance of gut microbiota

The Gastrointestinal tract (GIT) is a home to a complex and diverse microbial ecosystem whose composition varies between the habitant and regions of the gastrointestinal tract. This provides essential stimuli for the

host's immune system development and physiology such as gastric development, differentiation and integrity (Merrifield *et al.,* 2010a)

The intestinal microbiota does not exist as an organism alone, but the ecosystem and host functions are actively interacting (El-Haroun *et al*., 2006). The primary role of microbiota (an enormous number of microorganisms) involves degrading feed components providing nutrients and lipids by microbial metabolism, which provide essential nutrients that protect against invasive pathogens (Mulder *et al*., 2009). For example, *Lactobacilli* species are members of the indigenous microbiota which can produce bacteriocins known for their antibacterial actions. Hence, intestinal microbiota stability is very essential for the organism's health (Rollo *et al*., 2006).

Fish gut microbiota has been suggested to have positive effects on the digestive processes of fish and a wide range of enzyme-producing microbes have been isolated and identified. Other suggested potential contributors in addition to *Bacillus*, are *Microbacterium*, *Micrococcus*, unknown anaerobes and yeast. In addition, however, due the the complexity and variable ecology of the digestive tract of different fish species, it is difficult to conclude the exact contribution of GI microbiota to endothermal animals stomach and pyloric caeca presence (or absence) and relative intestinal length (Ray *et al.,* 2012).In particular, the microbiota of fish has a very significant and diverse enzymatic potential. It can produce proteolytic, amylolytic, cellulolytic, lipolytic and chitinolytic enzymes. These are essential for the digestion of proteins, carbohydrates, cellulose, lipids and chitin, especially some pathogenic compounds (Bairagi *et al*., 2002; Gutowska *et al.,* 2004). The enzymes that are produced by microbiota, particularly in the larval stage , can be beneficially used as probiotic supplements when formulating the fish diet. It provides fish nutrition with

the opportunity to use the enzyme producing isolates as a probiotic in cost-effective formulated diets for fish. However, more studies should be conducted to determine whether the addition of such isolates to fish feeds actually provides some sort of advantage to the fish concerned before their use is proven (Bairagi *et al*.,2002).

Furthermore, GI microbiota, on the other hand, may enhance the absorption of nutrients, particularly in the metabolism and transferring of cholesterol (Rawls *et al.,* 2004; Bates *et al.,* 2006). In a few fish species, the potential impact of gut microbiota on metabolism was examined. Many bio-syntheses and metabolism pathways of carbohydrates, amino acids and lipids alter in grass carp (*Ctenopharyngodon idella*) due to changes in the structure of microbiota modification (Ni and Yu, 2014). In zebrafish, intestinal colonization by microorganisms encourages the epithelial intake of fatty acids (Semova *et al.,* 2012) and fish with intact microbiota have reduced lipid accumulation in the intestinal epithelium and enhanced lipid metabolism gene expression compared to germ-free fish lacking microbiota (Sheng *et al*., 2018).

It is essential that once the organism has hatched, the GI tract is colonized to assist in the growth and development of not only body mass but also epithelial development and skeletal development (Nayak, 2010a). Colonization is easy when the fish begins to feed as the digestive tract is a major route of infection in fish (Ringø *et al*., 2006).

Probiotic microorganisms as a part of microbiota can increase the length of the microvilli in the intestinal tract (Al-Hisnawi *et al*., 2019) and this can enhance the host organism's nutrient absorption and boost the feed conversion ratio(FCR) and reduce the waste product in business terms (Pieters *et al.,* 2008). In their inquiry, the use of probiotics may decrease the

impacts of epidermal diseases and the capacity of probiotics to improve immunity against many disease outbreaks (Nayak, 2010a).

1-2-4 Definition of probiotic

The term “probiotic” comes from Greek words *pro* and *bios* meaning “for life” (Schrezenmeir and De Vrese, 2001). The probiotics are a live microbial feed supplement, which beneficially affects the host by improving its intestinal balance (Moriarty *et al* ;2005).The probiotics which are thought to have a broad range of positive impacts for the host animal, including increased immune response to pathogens, improving the development, the body composition and optimization of gut and microbial balance (Merrifield *et al.*, 2010b).

Another definition for probiotics was formulated by(Merrifield *et al*., 2010b) is "a live, dead or component of a microbial cell that when administered via the feed or to the rearing water benefits the host by improving its microbial balance or microbial balance of the ambient environment"

Probiotic has been identified as a live, dead or portion of a microbial cell that has beneficial effects on the host by improving disease resistance, growth efficiency, feed use and health status by achieving microbial balance in both the host and the environment (Hai, 2015).

1-2-5 Characteristics of probiotic

A high-quality probiotic should have the following mentioned characteristics (Fuller, 1989).

-Should be a strain that is capable of exerting a beneficial effect on the

host animal, e.g. increased growth or resistance to disease. Should not have any side effect; should neither be pathogenic nor toxic, not only with regard to the host but also with regard to aquatic animals in general and human consumers.

-Should be viable under normal storage conditions and able to survive during the industrial process.

-Should be capable of surviving and metabolizing in the gut environment, e.g. resistant to bile and low pH due to organic acids enrichment.

-Possess a high ability to multiply in the intestine.

-Possess strong adhesion ability with the gut of the fish.

-Should have strong antagonistic activity against pathogenic microorganisms.

1-2-6 Characteristics of *Bacillus. subtilis*

Bacillus signifies a gram-positive, rod shaped, spore-forming, aerobic or facultative anaerobic bacterium. In general, the genus *Bacillus* is designated as a group of soil inhabitants. However, *Bacillus* spp. can be isolated from varied sources including air, water, human and animal gut, and also from vegetables and food (Alou *et al.*, 2015; Kotb, 2015). *Bacilli* under the phylum Firmicutes represented in Figure (1-1).

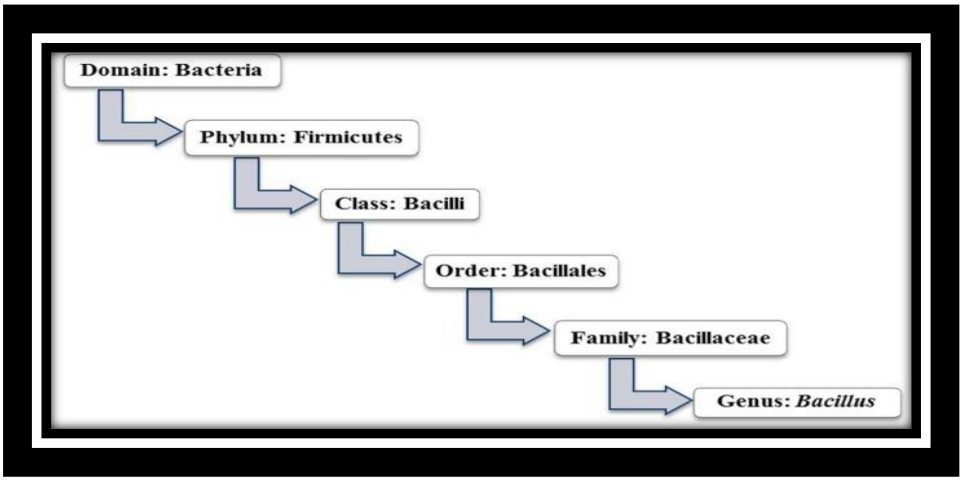


Figure (1. 1)Taxonomy of genus *Bacillus*(Bergey, D. H., & Garrity, G. 2001)

The spore forming *Bacillus* spp. and yeasts are the most commonly employed probiotic. *Bacillus* spp. have been shown to possess capacities of adhesion, produce bacteriocins (antimicrobial peptides) and provide immunostimulation (Cherif *et al*., 2001; Barbosa *et al.*, 2005).

For less than 50 years, the species of *Bacillus* including *B. subtilis*, *B. clausii*, *B. cereus*, *B. coagulans*, *B. circulans* and *B. licheniformis* have been used as a probiotic (Cutting, 2011; Farzanfar *et al*., 2009 Bandyopadhyay and Mohapatra , 2009). *B. subtilis* is between the oldest species on earth, reason why animals and humans since the beginning of their existence have been in contact with this bacterium. In this sense, recognition of *B. subtilis* for animal and human immune system is well established and a symbiotic relationship had been developed for a long period of time (Nicholson , 2004; Vreeland *et al* .,2000;cutting, 2011)

Bacillus subtilis spores are capable of resisting extreme pH circumstances, UV irradiation, elevated temperatures, solvents and lengthy refrigeration -free storage times (Olmos& Paniagua-Michel 2014) . It is, however, important to note that *B. subtilis* has (a) nutrient usage versatility, (b) elevated enzyme output, (c) antimicrobial secretion, (d) is a spore producer, (e) develops in aerobic and anaerobic conditions and (f) is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA) represented in Figure (1-2) . Based on these observations *B. subtilis* could be regarded as an ideal multifunctional probiotic bacterium for humans and animals in "theory" (Ochoa SJL, 2012 ; Olmos SJ *et al*., 2014; Huang Q. *et al.*, 2013). *B. subtilis* is not harmful to mammals, including humans, and is commercially significant as a producer of large and varied quantities of secondary metabolites such as antibiotic, chemicals and enzymes, as well as heterologous proteins, antigens and

vaccines (Olmos& Paniagua-Michel 2014 ; Bérdy, 2005; Valdez, *et al.*, 2014).

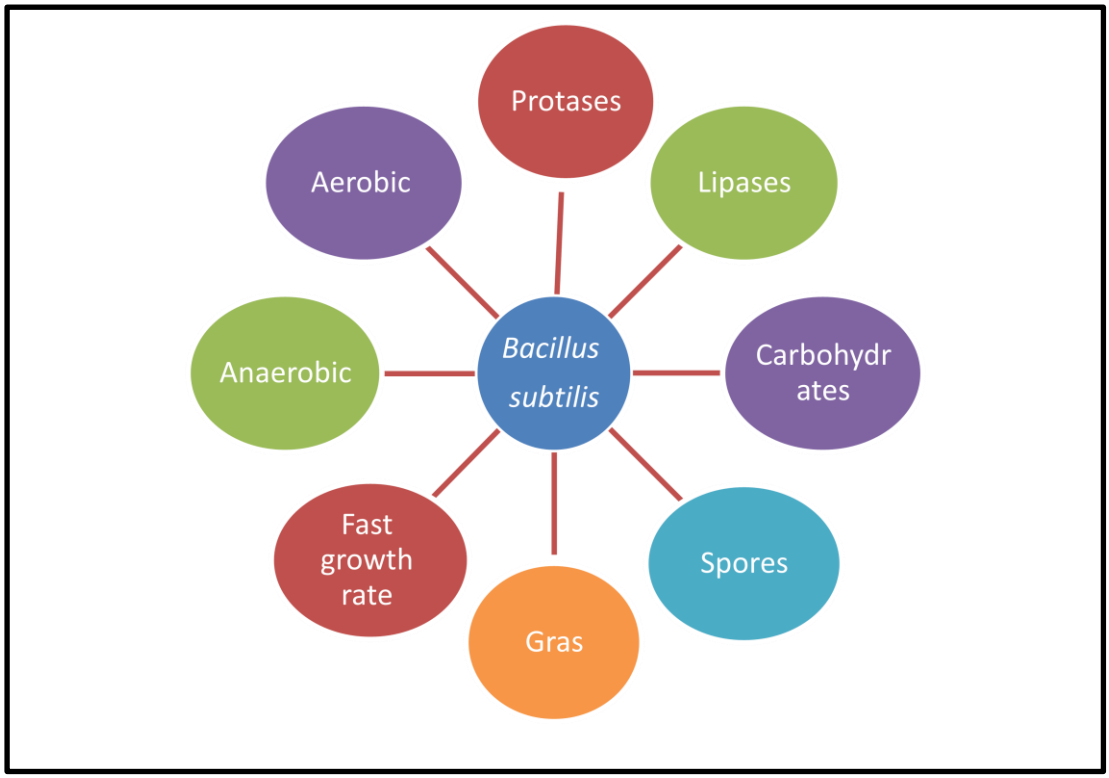


Figure (1. 2) Intrinsic properties of *B. subtilius* (Olmos, J., & Paniagua-Michel, J. 2014).

It is commonly stated that *B. subtilus* grow effectively with low-cost sources of carbon and nitrogen because their enzymes are very effective in breaking down a wide range of animal and vegetable proteins, carbohydrates and lipids into their constituent units (Sonenshein and Losick,. (1993 ; Olmos and Paniagua-Michel, 2014 ; Arellano and Olmos, 2002). The enzymes also degrade organic accumulated debris from dead shrimp and fish thereby inducing bioremediation in lakes and therefore prevent viral and bacterial diseases (Olmos *et al.*, 2011; Paniagua-Michel *et al.*, 2005). Since the genus was not reported as the pathogen in water organisms, it is widely used in an aquatic culture which can generate

antibiotics, amino acids, enzymes and amounts of peptides, including the subtilisin and bacitracin (Bagheri *et al.*,2008).

Probiotics including *Bacillus* spp. (Gram-positive endospore-forming bacteria) to monitor and interact with pathogenic bacteria can be introduced into the culture environment as well as supporting the growth of the animals cultivated. Additionally, *Bacillus* spp. when administered to aquatic organisms, are non-pathogenic and non-toxic microorganisms without adverse side effects (Farzanfar, 2006; Das *et al*., 2013).

Bacillus strains have been reported to be ideal for aquaculture as probiotics as they are commonly found in the gastrointestinal tract of animals as part of their microbiota. Some studies have reported that *Bacillus subtilis* enhances the growth of tilapia (Günther and Jiménez-Montealegre. 2004).

1-2-7 Modes of application of probiotics in aquaculture

Probiotics can be applied through feeding, injection, immersion or directly by adding in the water (Irianto, and Austin 2002b).

1-2-7-1 Application in feed

Probiotics are usually used by adding ingredients directly to the prepared feed. In aquaculture, *Lactobacillus* spp., and *Bacillus* spp., are widely used as probiotics (Robertson *et al*., 2000). According to the Food and Agriculture Organization (FAO) and World Health Organization (WHO) guidelines, probiotics should be able to survive through the intestine as well as resist gastric juices and bile salts. In addition, probiotics should be capable of flourishing and settling in the intestine, which should be secure and efficient for the host species (Senok *et al*., 2005).

1-2-7-2 Application through immersion or injection

It is also possible to use probiotics by injecting or immersing (Austin, 1995; Yassir *et al*., 2002). Earlier research showed that the impact of intra-peritoneal injection of a well-known probiotic *Micrococcus luteus* to *Oreochromis niloticus* (Nile tilapia) resulted in only 25 percent mortality while 90 percent death with *Pseudomonas* was observed(Akter *et al* .,2016).

1-2-7-3 Direct application to culture water

The Probiotic application directly in lakes and tanks also had positive effects on fish health by modulating water and sediments' microbial structure and water quality (Venkateswara, 2007). Probiotic bacteria, *Bacillus* spp. in particular and some species including *Aerobacter* spp., *Nitrobacter* spp. and *Saccharomyces cerevisiae* (yeast) performed a great work in improving water quality and gram-negative bacteria are capable of transforming a bigger quantity of organic substance into bacterial biomass or slime (Mohapatra *et al*., 2013; Kolndadacha *et al*., 2011).

1-2-8 Mechanisms of action

Literatures has shown the possible mode of action of probiotics in aquaculture which include the production of inhibitory compounds, improvement in nutrient digestion, colonization activity, enhancement of immune response, competition for nutrients (Defoirdt *et al.*, 2007; Martínez Cruz et al., 2012), improving water quality (Hai, 2015), and antiviral activity (Zorriehzahra *et al.*,2016 ; Idowu, and Sogbesan,. 2017).Represented in Figure(1-3)

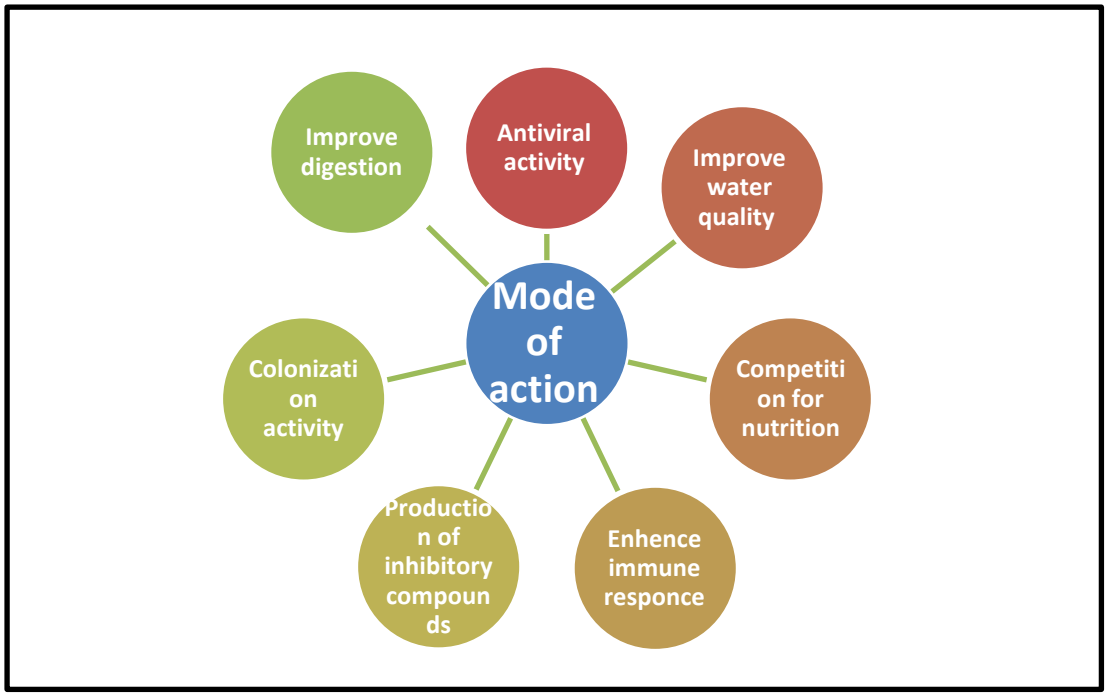


Figure (1. 3)Mechanisms of action of probiotic microbes (Al-Hisnawi *et al.*, 2015).

1-2-8-1 Production of inhibitory compounds

Bacterial antagonism is a prevalent occurrence in nature therefore, in the balance between competing useful and possibly pathogenic microorganisms, microbial interactions play a significant role (Balcazar *et al.*, 2006). One of the suggested processes engaged in probiotic health advantages involves production of low molecular weight (LMW), compounds (< 1,000 Da), such as organic acids, and the manufacture of antibacterial drugs known as bacteriocins (>1 1,000 Da). Organic acids, specifically acetic acid and lactic acid, have a powerful inhibitory impact on gram-negative bacteria responsible for probiotic- pathogens inhibitory activity (Alakomi *et al.*, 2000; Makras *et al.*, 2006). It is noteworthy that probiotic bacteria produce a wide range of chemical compounds in the host's intestine, including bacteriocins, siderophores, lysozymes, proteases and hydrogen peroxide. The production of organic acid will lead to the inhibition of the proliferation of opportunistic pathogens and modification of intestinal pH (Korkea-aho *et al.*, 2011;Perez-Sanchez *et al.*, 2011). Many

lactic acid bacteria (LABs) produce antibacterial peptides, including bacteriocins and tiny antimicrobial peptides (AMPs) Gram-positive bacteria (generally LAB, including *L. acidophilus* lactacin B, *L. plantaricin*, *L. plantarum* and *Lactococcus lactis*) have a narrow spectrum of activity and only act against tightly associated bacteria, but some bacteriocins are also effective against food-borne pathogens (Nielsen *et al*., 2010). Nisin forms a complex with the ultimate precursor to the cell wall, lipid II, thus inhibiting the biosynthesis of mostly spore-forming bacilli. The complex then aggregates and combines peptides into the bacterial membrane to form a pore (Bierbaum and Sahl, 2009).

Lastly, probiotic bacteria can generate so-called de-conjugated bile acids that are bile salt derivatives. De-conjugated bile acids have a greater antimicrobial activity relative to the host organism synthetic bile salts. It continues to be clarified how probiotics defend themselves against their own bactericidal metabolites or whether they are at all resistant to deconjugated bile acids (Oelschlaeger, 2010).

1-2-8-2 Antiviral activity

Viral fish diseases are difficult to diagnose and control with medication (Francis- Floyd & Wellborn, 2005). Other viral fish diseases include infectious pancreatic necrosis (IPN), hematopoietic infections channel catfish virus disease (CCVD), and viral haemhorragic septicaemia (VHS). The best measure is to take control of preventing viral infections. Vaccines rarely control Virus disease of fish (Bassey, 2011).

It is generally accepted that the antiviral activity of probiotics has increased (Lakshmi *et al*., 2013)**.** *Pseudomonas*, *Vibrio, Aeromonas* spp. and Coryneforms for example, had antiviral activity against infectous hematopoietic necrosis virus (IHNV) (Kamei *et al.,* 1988). Furthemore,(Li

et al., 2009) . Showed that *Bacillus magisterium* strain feeding increased resistance to white spot syndrome virus (WSSV) in *Litopenaeus vannamei* shrimp. It was documented that probiotics such as *Bacillus* and *Vibrio* sp. positively protect shrimp *L. vannamei* against WSSV **(**Balcazar, 2003).

1-2-8-3 Competion nutrition

Survivail of any microbial species depends on their ability to compete with other microbes in the same ecosystem for chemicals and available resources (Verschuere *et al*., 2000). Many microorganisms, recognized in the probiotic group known as lactic acid bacteria, consume the nutrients necessary for the growth of a number of pathogens (Brown, 2011). They are also competing for essential nutrients and enzymes resulting in enhanced host nutrition and the modulation of interactions with the environment and the development of beneficial immune responses (Ringø *et al*., 2010). Probiotic bacteria ferment food from indigestible starch to create short chain fatty acids in the intestine. The short fatty acid chain allows the systemic blood lipid levels to decrease by inhibiting synthesis of hepatic cholesterol and/or transfer of plasma cholesterol to the liver suggesting improved health status in fish (Tukmechi *et al.*, 2007).

1-2-8-4 Colonization activity

Adhesion is the preliminary step for colonisation of epithelial surfaces inthegut by bacteria and could be a criterion for selecting host microbiota to find the bacteria that are suitable as probiotic in fish farming (Grześkowiak *et al.*,2011). Microbes are more likely to colonize the mucosal epithelia when they have an ability to survive there for a long period, and by having are production rate, which is higher than their exclusion rate (Ringø *et al.*,2003). Different strategies are shown as passive

forces, electrostatic interactions, hydrophobic, steric forces and lipoteichoic acids in the adhesion of microorganisms to the attachment sites (Salyers and White, 2002).

Lactic acid bacteria (LABs) show different surface determinants involved in their association with intestinal epithelial cells (IECs) and mucus. Secreted mucin by IECs is a complex glycoprotein mixture which is the main component of mucus and thus prevents the adherence of pathogenic bacteria (Collado *et al* ., 2005; González *et al*., 2012). Recent studies have demonstrated that probiotic bacteria have an ability to adhere to the GI mucosa (Dicks and Botes, 2010; Merrifield *et al.*, 2010a). Bacterial colonization is influenced by a variety factors, which not only relate to the host, such as body temperature, enzymes, bile salts, gastric acidity,digestive enzymes, immune parameters, but also relate to (inhibitory)compounds produced by autochthonous microbes, such as proteases,bacteriocins, lysozymes, ammonia, hydrogen peroxide and organic acids that lead to changes in pH (Balcázar *et al.*, 2006) .

1-2 -8-5 Improved water quality

In various approaches, probiotics have demonstrated their effectiveness in improving water quality. They increased organic matter decomposition, decreased levels of nitrogen and phosphorus, and regulated ammonia nitrite and hydrogen sulphide. In addition, probiotics revealed decrease in sediment phosphate pollution and improved prawn farm environmental circumstances (Hai, 2015). Gram-positive bacteria, such as *Bacillus* spp., are used to enhance the water system quality. *Bacillus* spp. have more powerful skills when turning organic matter into carbon dioxide compared to gram-negative bacteria, which convert a larger proportion of organic matter into bacterial biomass or slime However, probiotics are beneficial because they can increase the concentration of microbial species

in the water and improve their quality (Balcazar *et al.,* 2006; Mohapatra *et al.,* 2013). By adding probiotics, temperature, pH, dissolved oxygen, NH_3 and H_2S were found to be of higher quality, thereby maintaining a good healthy environment for shrimp and prawn larvae in green water system (Banerjee *et al*., 2010; Aguirre-Guzman *et al.,* 2012).

Microbial cultures (including *Vibrio* sp. and *Aeromonas* sp.) produce a number of enzymes including amylase, protease, lipase, xylanase and cellulase at high concentrations compared to native bacteria which help degrade the waste. These bacteria have a wide range of salinity, temperature and pH tolerances.The phenomenon of this biotechnology philosophy recognizes is that the improvement of water quality is not from water or shrimp, but bacterial populations. (Hemaiswarya *et al.,* 2013).

Many probiotic strains are more effective in reducing through transforming organic matter or large polymers into smaller units, organic load in the aquatic environment. It also releases free amino acids and glucose through accelerating the rate of breakdown of organic matter, providing food sources (Balcázar *et al*., 2006).

1-2-8-6 Improve digestion

In digestion process of aquatic animals, some microorganisms have a beneficial impact (Balcazar *et al.,* 2006). Some bacteria have been shown to contribute to the digestion cycle through extracellular production of enzymes such as protease, lipases, and factors promoting growth (Wang *et al.,* 2000). Previous studies showed that some probiotics, particularly from *Bacteroides* and *Clostridium* sp., can supply vitamins, fatty acids, and critical host amino acids (Balcazar *et al.,* 2006; Tinh *et al*., 2008).

Carbohydrate, fat and protein digestion are primarily dependent on different types of enzymes such as amylase, lipase and protease,

respectively. There is evidence of increased feed use and growth of Nile tilapia following the feeding of probiotic diets by increasing starch, fat and protein digestion (Essa *et al*., 2010). Increased digestive enzyme activity including amylase, lipase, and protease was also noted in *Labeo rohita* fed with a combination of three probiotics such as *Bacillus subtilis, Lactococcus lactis, and Saccharomyces cerevisiae (*Mohapatra *et al*., 2013)

1-2-8-7 -Enhanced immune responses

Probiotics can enhance the immune system of hosts, including the development of immune cells by inducing pro-inflammatory cytokines and increasing the phagocytic role of leucocytes on the function of immune cells (Pirarat *et al.,* 2006), increasing antibody levels, activity of lysozyme enzyme (Lara-Flores & Aguirre-Guzman, 2009), complement activity (Balcazar *et al.,* 2007), interleukin-1 (IL-1), IL-6, IL-12, tumor necrosis factor α (TNF-α), interferon gamma (IFN-ɣ) (Nayak, 2010) and antimicrobial peptides (Mohapatra, *et al.,* 2012). It is found that *B. subtilis* control pro-inflammatory cytokines such as IL-1β1 and TGFβ in the spleen and head kidney of rainbow trout *Oncorhynchus mykiss* (Panigrah *et al* ., 2007).

The study of (Nurmi *et al*., 2005) reported down-regulation of L-transcripts of Cyclooxygenase 2 (Cox-2) along with TGF-β and IL-10 genes in *Dicentrarchus labrax* fed diet supplemented with *Lactobacillus delbrueckii*. It is thought that COX-2 facilitates the healing of intestinal wounds but its chronic expression can lead to inflammatory diseases.Several studies have documented the ability of the administered probiotic to modulate non-specific immune responses and increase disease resistance during bacterial infections in aquatic animals (Balcazar *et al.,* 2006; Gatesoupe, 2008). Moreover, the number of leucocytes in probiotics (lymphocytes, monocytes) and erythrocytes count have increased, with

neutrophilic adherence, neutrophilic migration and bactericidal plasma activity, complementary activity (Heo *et al.*, 2013). The use of *Bacillus* sp. was investigated to provide disease resistance by stimulating immune defenses both cellular and humoral in tiger shrimp (*Penaeus monodon*) (Balcazar, 2003).

1-2-9 The disease resistance methods in fish (immunity)

Like other vertebrates, the fish immune system can be divided into innate (non-specific) immunity and adaptive or (specific) acquired immunity. It is possible to distinguish both innate and adaptive immune response into cell-mediated and humoral (soluble) response factors. Innate immune response precedes adaptive immune response as an initial line of defense against infection, activating it and modulating its existence. Innate and adaptive immune responses are working together to preserve homeostasis and are therefore considered a mix of systems (Magnadóttir, 2006). Innate immune system generally responds to a wide range of foreign stimuli and is activated in the host body immediately or within hours of the appearance of the pathogen. It is therefore a crucial factor in resistance to disease (Secombes and Wang , 2012).

The adaptive response is typically delayed, but is critical for long-term immunity due to its ability to produce immunological memory, and is therefore a key factor in vaccine development and preventive function (Sallusto and Lanzavecchia, 2009; Secombes and Wang, 2012) .It is believed that two categories of molecular patterns induce an immune response: foreign or pathogenic molecular patterns and molecular patterns that are exposed by damage to the host's own tissues, because of infection, necrotic changes and natural death of cells, signalling danger to the immune system. For example, the molecular patterns of these parameters are

peptidoglycans and bacterial cell wall lipopolysaccharides (LPS), fungal β1,3-glucan, viral double-stranded RNA, and bacterial DNA. Pathogen-associated molecular patterns (PAMP) is the collective term used for these highly conserved molecules not commonly expressed in multicellular organisms (Elward,and Gasque 2003).

1- 2-10 Cellular components of fish innate immune system

A number of cell types, including macrophages, granulocytes (e.g. neutrophils), non-specific cytotoxic cells (NCC) and natural killer (NK)-like cells, are actively involved in fish innate immune response. In innate immune systems, mast cells and rod let cells also play an important role in immune response (Secobes and Wang, 2012; Firdaus-Nawi and Zamri-Saad, 2016).

1-2-11 Humoral factors of fish innate immunity

Humoral causes include cell receptors or other chemicals, such as plasma, which are soluble in body fluids. Innate humoral defense of teleost fish includes different bacterial inhibitors growth, for example: transferrin, lysozyme, C-reactive protein, alkaline phosphatase, antimicrobial peptides, supplementation, lectins (Magnadottir, 2006).

1-2-12 Cytokines

Cytokines are tiny, soluble pleiotropic proteins that nearly all of the body's cells secrete and have both autocrine and paracrine functions. They function by binding specific membrane receptors and by signaling other genes, usually those involved in cellular activation, growth or differentiation, to be transcribed. Nonetheless, the primary role of cytokines is to modulate the immune response. There are two major groups of cytokines in inflammation, the pro-inflammatory cytokines secreted by

activated macrophages and the anti-inflammatory cytokines which are involved in inflammatory response (Pressley *etal*,. 2005).

Probiotics such as *Bifidobacterium, Lactobacillus acidifilus*, *Lactobacillus paracasei* and *Lactobacillus plantarum* can regulate the expression of different types of cytokines in different hosts (Rutherfurd-Markwick& Gill, 2004). Different strains of LAB can induce regulatory and pro-inflamatory cytokines while other probiotics can increase intestinal inflammatory responses (Perdigon *et al.,* 2002). Probiotics such as *Lactobacillus rhamnosus, Enterococcus faecium* and *B. subtilis* have revealed controlling of pro-inflammatory cytokines such as IL-1β and TGFβ in spleen and head kidney of rainbow trout (*Oncorhynchus mykiss*) (Panigrahi *et al.,* 2007).

The spores of *B. subtilis* stimulate specific humoral and cellular activity (Amuguni and Tzipori, 2012). Interaction with *B. subtilis* showed that spores play a major role in creating the both innate and adaptive immune responses of the host (Guo *et al.,* 2017). In addition (Suva *et al*., 2016) declared that *B. subtilis* B10, *B. subtilis* BS02, and *B. subtilis* (natto) B4 spores may have immunomodulatrey function, given by induction of pro-inflammatory cytokines which exercise probiotic activity functions activated for macrophage. *B. subtilis* promotes active proliferation of lymphocytes within the gasterointestinal tract (GIT), as a result of high metabolic activity.

Bacillus activity is determined mainly by their ability to produce antibiotics of which, the most important species is *B. subtilis Bacillus subtilis* devotes 4-5 % of its resources to Antibiotic synthesis, and generates as many as 66 antibiotics. Each antibiotic *Bacillus* contains a

similar antimicrobial composition and spectrum of activities (Sorokulova, 2013).

1-2-12 -1 Interleukin one beta (IL1β)

Interleukin one beta (IL1β) is a well-characterized cytokine and plays an important role in addressing immunological risks, infection and inflammation in cellular responses. Macrophages are the primary source of IL1β, although they are secreted by various types of cells; natural killer (NK) cells, B cells, Langerhans skin cells, peripheral neutrophilic granulocytes, endothelial cells, fibroblasts, and microglia cells are included (Huising *et al.*, 2004; Tassakka and Sakai, 2004). IL1β is a key player in the defense against invasion of microorganisms and tissue injury and can trigger immune responses by stimulating lymphocytes or by enhancing the release of other cytokines capable of activating NK cells, macrophages and lymphocytes (Low *et al.*, 2003). Among fish, IL1β is expressed constitutively in several tissues, such as spleen, head of kidney and liver, and increased spleen expression has been observed (Tafalla *et al.*, 2005; Lu *et al.*, 2008).

1-12-2 Transforming growth factor β (TGF-β)

Transforming growth factor β (TGF-β is a pleiotropic cytokine that regulates the growth, proliferation, differentiation, migration and survival of cells in different leukocyte lines, including lymphocytes, dendritic cells, NK cells, macrophages and granulocytes (Li and Flavell, 2008 ; Li *et al* ., 2006). TGF-β1 is a well-known suppressive cytokine in the mammalian immune system and has a dominant role in maintaining immune tolerance and suppressing autoimmunity (Saxena *et al*., 2008; Zhang *et al*., 2006). TGF-β has powerful immunosuppressive effects mediated primarily through its multiple effects on T cells: TGF-β suppresses Th1 and Th2 cell

proliferation, in mammals the regulatory roles of TGF-β as a positive or negative immune control device are widely recognized (Li and Flavell, 2008; Li *et al*., 2006; Wan and Flavell, 2007).

In teleosts, despite the lack of extensive investigation into the functional role of TGF-β, several studies have shown that TGF-β often exerts powerful immune-depressive effects on activated leukocytes, as it does in mammals. For example TGF-β1 pointedly blocks TNF-α-induced activation of macrophages in goldfish and common carp, but TGF-β induces goldfish fibroblast cell line CCL71 proliferation (Haddad *et al*., 2008; Kadowaki *et al.,* 2009). In grass carp, TGF-β down-regulates lipopolysaccharide (LPS) phytohemagglutinin (PHA) LPS / PHA stimulates perppherial blood lymphocyte profileration as opposed to TGF-β stimulating effect alone in the same cells (Yang *et al*., 2010). Similar phenomenon was observed with or without LPS challenges during leukocyte migration under TGF-β treatment in red sea bream (Cai *et al* ., 2010).

Chapter Two

Materials and methods

2- Materials and methods

2-1 Materials

2-1-1Apparatus and instruments

The equipment used in the present study with their manufacturing companies are given in the Table (2-1).

Table (2. 1) Apparatus and instruments.

No	Apparatus /instruments	Company name /origion
1-	Autoclave	Lab Tech –Korea
2-	Cooling centrifuge	Beckman-USA
3-	Distillator	Daihanlab tech – Korea
4-	ELISA reader biotic	Biotic -U.S.A
5-	EDTA tubes	P.R- China
6-	Eppendorf tube (1.5ml)	Merk-Germany
7-	Gel tubes	Citotest- China
8-	Glass thermometer	Mart -India
9-	Gram stain kit	AL Hannof- Jordan
10-	Incubator	Daihanlab tech - Korea
11-	Microscope	Olympus BX41-Japan
12-	Micro pipette 100-1000 µl and 10-100 µl	Biopette plus - Labnet
13	Nanodrop	Cambridge(United kingdom)
14-	PCR tubes (0.3 ml)	Eppendrof – Oxford
15-	pH meter 211	Hanna – China
16-	PCR 080725 Multigene	Labnet International Inc
17-	Refrigerator	Concord- Lebanon
18-	Shaking incubator	D aihan lab tech- Korea
19-	Spectrophotometer	Shmadzu-Japan
20-	Sensitive balance	Sartorius –Germany
21-	UV transilluminator	Syngene –England
22-	Ultra violet translinker -TL -2000	UVP- England
23-	Vortex	Huma twist- Germany
24-	Water bath	Tafesa – Germany
25-	Filter paper	Ahlstrom-Sweden

2-1- 2 Cultures media used in this study

Table (2- 2) Cultures media used in this study

NO.	Name of media	Company name/origion
1-	Blood agar	Oxoid- Canada
2-	de Man, Rogosa and, Sharpe (MRS agar	Oxoid – England
3-	de Man, Rogosa and, Sharpe (MRS Broth	Oxoid –England
4-	Eosin methylene blue agar (EMB)	Himedia – India
5-	Brain heart infusion(BHI)	Oxoid –England
6-	Nutrient broth (NB)	Himedia –India
7-	Tryptone soya agar (T.S.A)	Himedia – India

2-1-3 Biological and Chemical materials

Table 2. 3 Biological and chemical materials used in the present study and their manufacturing companies.

Biological and Chemical materials	Company Name/origion
Agarose	Bio basic- Canda
Absolute ethanol(99%)	Algoid –Iraq
Glugose	Afco-India
Maltose	Sigma-India
Manitol	Sigma-India
Manose	Sigma-India
Clove oil	Gopaldas visram&coltid-India
Fish oil (cod liver oil)	H&R laboratories – Pakistan
H_2O_2(3%)	BDH-India
Iodine solution	AL Hannof-Jordan
Ethanol (70%)	Algoid- Iraq
Kovacs reagent	CDH-India
Nuclease-free water	BioLabs Inc. New- England U.S.A

Peptone	Himeda- India
Tris-Borate-EDTA buffer (TBE buffer) 0.5X	Bio basic Inc.- Canada
Loading Dye	Intron- England
Red Safe™	Intron- England
Crystal violet	Al -Hannof Jordan
Safranin	Al -Hannof Jordan
Malachite –Green	Himeda-India
Tetra methyl p-Phenylenediamine dihydrochloride(oxidase reagent)	Himeda –India

2-1-4Kits

The kits used in the current study with their manufacturing companies are presented in Table (2-4).

Table (2-4) Kits used in this study with their origins.

Name of kit	Storage	Name of company and origin
DNA extraction	At room temperature	Intron biotechnology Inc. – Korea
Favorv PREP ™ PCR Clean –Up Mini	At room temperature	Favorgen Biotechnology crop- Korea
DNA Ladder	4 ºC	Intron – Korea
PCR Master Mix	-20 ºC	Bio labs- England
Fish Transforming	2-8 °C	Bioassay technology laboratory- China

Growth Factor Beta ELISA Kit		
Interleukin 1β ELISA Kit	2-8 °C	Bioassay technology laboratory- China
API 20 E kit	2-8 °C	Biomerieux- France

2-1-5Primers

Primers used in this study were synthesized by Humanizing Genomics Macrogen Korea. The name, sequence and product size are given in Table (2-5)

Table (2. 5) The name sequence and product size of primers used in this study for16 *rRNA* designed in this study

Name of primer	**Sequence of primer 5'-3'**		**Product Size (bp)**
27F	F	5'AGA GTT TGA TCC TGG CTC -3	1400
1492R	R	5 GGT T AC CTT GTT ACG ACT T- 3	

2-2 Methods

2-2- 1 Preparation of culture media

2-2-1- 1de Man Rogosa and Sharpe Agar (MRS)

It was prepared according to the instructions of the supplied company by dissolving 13 g of the medium in 1 liter of distilled water then autoclaved at 121 ºC for 15 minutes after cool for 45- 50 ºC. About 20 ml of medium was distributed in each Petri dish, this medium was used for initial isolation of lactic acid bacteria (LAB).

2-2-1-2 Tryptone soy agar (TSA)

It was prepared according to the instructions of the supplied company by dissolving 40 g of the medium in 1 liter of distilled water then autoclaved at 121 ºC for 15 minutes after cool for 45- 50 After that 20 ml of medium was distributed in each Petri dish, this medium was used for initial isolation of total viable bacteria (TVC).

2-2-1-3 Nutrient Broth (NB)

It was prepared according to the instructions of the supplied company by dissolving 13.0 g of the medium in 1 liter of distilled water in flasks .The produced solution was distributed in the glass tubes, then autoclaved at 121 ºC for 15 minutes after cool for 45- 50 After that 2 This medium was used to activate the general bacteria.

2-2-1-4 Blood agar

It was prepared according to the instructions of the supplied company by dissolving 40 g of the medium in 1 liter of distilled water then autoclaved at 121 ºC for 15 minutes after cool for 45- 50 ºC. Human blood was added to the medium mixed gently after that poured into petri dishes. It is used for primary isolation and to detect the ability of bacteria to hemolysis red blood cells (RBCs).

2-2-1-5 Eosin Methylene Blue agar (EMB agar)

Was prepared according to the instructions of the supplied company by dissolving 36.96 g of the medium in 1 liter of distilled water .Heating in order to dissolve the media completely. then autoclaved at 121 ºC for 15 minutes after cool for 45- 50 ºC and left for cooling to 45 °C. Medium was shaken in order to oxidize the methylene blue (to restore its blue color) and to suspend the flocculent precipitate. Medium

was mixed well and poured into sterile Petri plates, this medium was used as selective media for gram negative bacteria.

2-2-1-6 Pepton water

Medium was used for detection the ability of indole ring production it was prepared according to the instructions of the supplied company by dissolving dissolving 20 g of peptone and 5 g of sodium chloride (NaCl) in a quantity of distilled water and after adjusting the pH to 7.4, completing volume to 1 liter with distilled water, then distributed the medium in sterile tubes.

2-2-1-7 Motility test medium

Medium was prepared according to the instructions of the supplied company by adding 1 g of agar-agar to 200 ml of (Nutrient broth). Heating in order to dissolve the media completely and distributed in glass tubes of 5 ml per tube and autoclaved at 121 ºC for 15 minutes .Tubes were left for harden vertically, this medium was used to test the movement of bacteria .

2-2-1-8 Sugars preparation medium

This medium was prepared by dissolving 10 grams of peptone, 5 grams of sodium chloride (NaCl) in a liter of distilled water and adding 50 ml of phenol red detector at a concentration of 0.2%, adjusted the pH to 7.2. The medium was sterilized by autoclave, and 4.950 ml was distributed in each sterile tube and then the filtered steril sugar (maltose, mannose, maltose, glucose, cellulose, and arabinose) were added after sterilizing them by using filters with a pore diameter of 0.45 micrometers. Sugars were prepared from the melt of 1 gram of sugar in a quantity of distilled water and after that the melting volume was completed to 100 ml

in a volumetric bottle and 50 µl from this solution was added to each tube (MacFaddin, 2000).

2-2-1-9 Brain heart infusion broth

It was prepared according to the instructions of the supplied company by dissolving 37.0 g of the medium in 1 liter of distilled water in flasks .The produced solution was distributed in the glass tubes, then autoclaved at 121 ºC for 15 minutes.used for fastidious and nonfastidious microorganism .

2-3- Preparation of Reagents

2-3-1 Oxidase reagent

Reagent was prepared by dissolving 1 g of tetramethyl p-phenylene-diamine dihydrochloride in 100 ml of distilled water into a sterile and dark bottle. This reagent was used to investigate the abililty of bacteria to produce an oxidase enzyme (Tang and Stratton, 2006).

2-4 Experimental design

The present study was carried out in the aquarium of College of Agriculture at Kerbala University-Iraq. Common carp fish were purchased from a local commercial farm at Kerbala city. The current trial lasted for six weeks from (24 February to 10 April 2019). Fish were transported in oxygenated polythene bags to the aquarium and left for two weeks before commencement of the trial for adaptation to the new environmental condition. A total of 70 fish (77.2 ± 0.86 g) were randomly distributed in 14 tanks (seven tank probiotic *bacillus subtilius* , and other seven tanks were allocated for the control group, each tank contain 5 fish). represented in the Figure (2-1).The tanks were thoroughly cleaned every two weeks when fish subjected to weighing and water waspartially

changed in the system if necessary. Manual system for photoperiod of 12h light and 12h dark was used throughout the trial.

Figure (2. 1) Aquarium used for the present experiment at College of Agriculture, Kerbala University.

2-5 Water quality

During the trial, water quality parameters such as temperature, dissolved oxygen (DO), and pH were measured. Temperature and pH in the system were measured daily using glass thermometer and pH meter. The water temperature was maintained at suitable temperature (22± 1°C) throughout the experiment, the pH maintained at the level within the desired range (6.5- 7.5) and dissolved oxygen levels were kept above 80% with additional aeration provided by a side supply of compressed air.

2-6 Protocol for preparing *Bacillus subtilis* concentration curve

1-One colony of Bacillus subtilis was added to conical flasks contain 350 ml of MRS broth.

2- Theses flasks were incubated at 37^{o}C for 24 hours.

3-At 24 hours the following 10 fold dilutions (in Bijoux bottles at a total volume of 1 ml) were made using MRS as the diluent:

10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}

4-One hundred µl of the dilutions made in step 3 was spread onto triplicate MRS plates.

5- The following dilutions (in Bijoux bottles at a total volume of 1 ml) were made using MRS broth as the diluent:

25µl (culture broth)/975ul(sterile broth), 75/925, 50/950, 100/900, 200/800, 300/700, 400/600, 500/500, 600/500, 700/300, 800/200, 900/100.

6- MRS was used to zero the spectrophotometer (at 590nm) and then the absorbance of the dilutions made in steps 3 and 5in Table (2-6) were recorded.

7-Count plates made in step 4 after 24-48 hrs were calculated CFU ml^{-1} and recorded in Tables (2-7) .

8- The absorbance values and CFU ml^{-1} values were used to construct a calibration curve.

Table(2-6) Absorbance at 590 nm

Replicate	25ul/975 ul	50/950	75/925	100/900	200/800	300/700	400/600	500/500	600/400	700/300	800/200	900/100
A	0.140	0.365	0.251	0.467	0.790	0.908	1.109	1.176	1.283	1.287	1.384	1.379
B	$1.42*10^8$	$2.82*10^8$	$4.27*10^8$	$5.7*10^8$	$11.4*10^8$	$17.1*10^8$	$22.8*10^8$	$28.5*10^8$	$34.2*10^8$	$39.9*10^8$	$45.6*10^8$	$51*10^8$
C	-	-	-	-	-	-	-	-	-	-	-	-
Average	-	-	-	-	-	-	-	-	-	-	-	-

Table(2- 7) Bacterial counts after 24-48hrs

Replicate	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Optical density	0.481	0.055	0.006	-0.002	-0.002	-0.00	0.003	-0.00	-0.00
A	-	-	-	-	-	-	250	50	92
B	-	-	-	-	-	-	278	65	87
C	-	-	-	-	-	-	268	56	138
Average	$5.7*10^8$	-	-	-	-	-	-	$5.7*10^9$	-

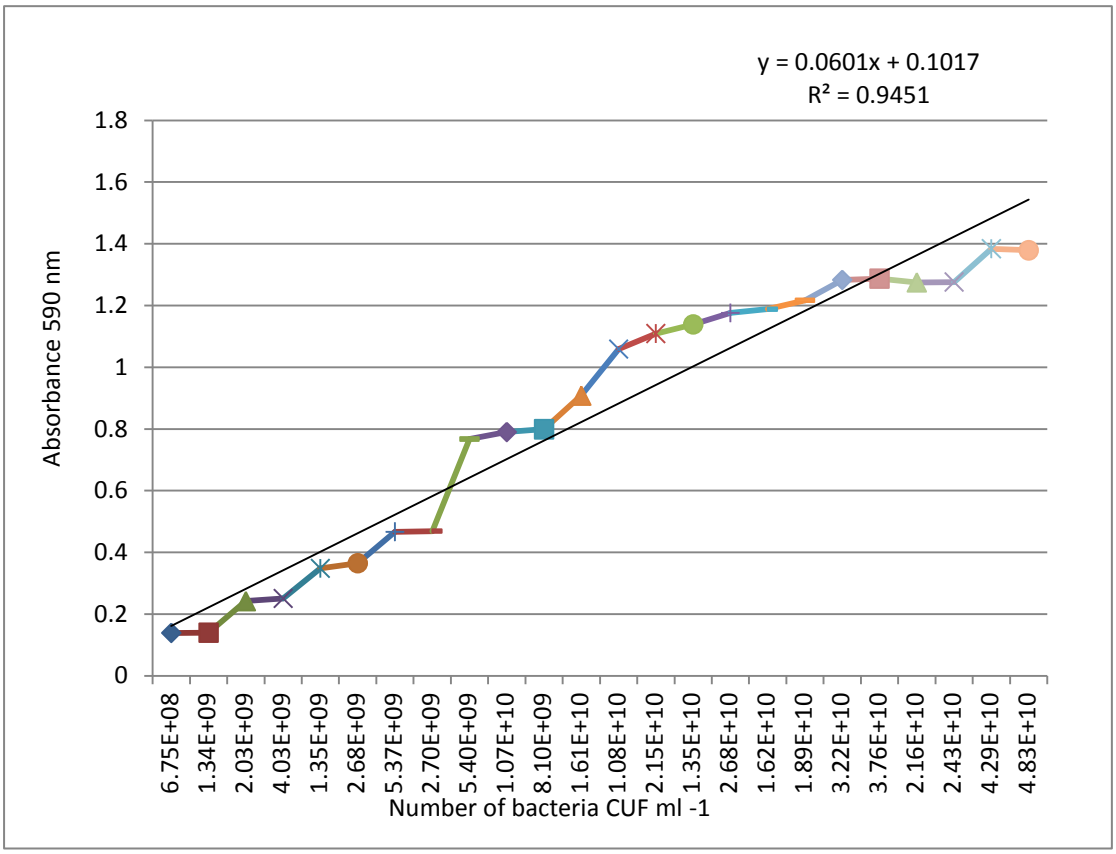


Figure2. 2 Standard curve for detection of the level of *B. subtilis* in the diet

Table(2.8) Ingredients of commercial diet componts used in the present study.

Composition components	**Ratio**
Crude fat	7 %
Potassium total	1 %
Fiber	6.1 %
Sodium total	0.2%
Calcium total	0.8%
Vitamin D3	1,000 IU /Kg
Vitamin A	10.000 IU /Kg
Crude protein	30%
Ca iodate	3mg /kg
Znic sulphate	70 mg /Kg

2-7 Feeding and weighing

Fish were hand-fed 1.5% of biomass twice daily provided in equal ratios at 09.00 and 17.00 for a period of six weeks. Daily feed was corrected on a fortnightly basis following batch weighing.

2-8 Microbiology sampling

Three fish from each group were euthanized at the end of the trial by immersion in clove oil (4) drops in 100 ml of water, followed by hard blow to the brain. Each fish was cleaned using 70% Ethanol to avoid possible external contamination while removing the intestine. Fish was dissected under aseptic conditions, and the intestine was completely excised. The intestinal tract was divided into two sampling regions, the anterior intestine and the posterior intestine.

After cutting at the proximal boundary between the two sections, the digesta of the anterior and posterior regions was obtained by gently squeezing using sterile forceps. Each digesta section was individually emptied into a sterile 1.5 ml eppendorf tube. Each section of the mucosa was aseptically opened longitudinally with a sterile scalpel and washed thoroughly three times with PBS . Each mucosa section was emptied into a Petri dish, thus four samples were obtained: anterior mucosa (AM), posterior mucosa (PM), anterior digesta (AD) and posterior digesta (PD). (Al-Hisnawi *et al*., 2015).represented in Figure(2-3)

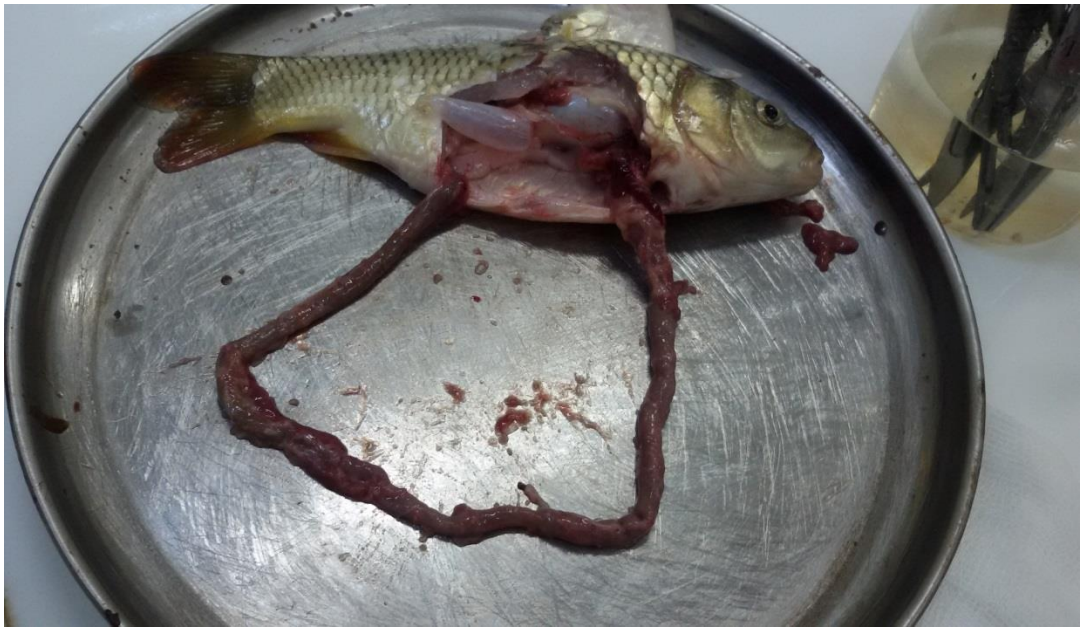


Figure (2.3) Regions of intestine in commn carp

2- 9 Isolation of bacterial populations

One gram (wet weight) of the sample material (mucosa and digesta samples) was homogenized using sterile tubes for 30 sec and vortexed vigorously for five sec in 9 ml of sterile PBS. Samples were tenfold diluted with sterile PBS and 100 µl of each dilution was spread onto duplicate TSA and MRS plates which were incubated at 22 °C for 3 and 5 days for calculating numbers of the total viable count TVC and LAB, respectively, in the samples by counting colonies (plates containing 30–300 colonies). (Al-Hisnawi *et al*., 2015).

2-10. Identification of bacterial isolates

Distinctive colonies were randomly selected from TSA plates containing 30-300 CFU according to shape, size, color structure, surface and edge of colonies. These cultures were then sub-cultured on TSA repeatedly until pure cultures were obtained (Al-Hisnawi *et al*., 2015). Some colonies failed to re-culture in spite of attempts to repeat culturing

these colonies on appropriate media. Pure colonies were grouped according to cell shape, motility and endospore formation (observed from Gram stain) in order to identify the selected bacterial isolates to genus or species level following the criteria described in Bergey′s Manual of Systematic Bacteriology. The isolates were stored at 4 °C. After that, the isolates were then identified more using biochemical tests in API 20E

2-10-1 Biochemical Tests

2-10- 1-1 Gram Stain

It was prepared according to the instructions of the supplied company**:**

1. Transfer adrop of the suspended culture to be examined on the a clean glass slide.
2. Dry the slide in the air, and then fixed heat
3. Crystal violet was poured and kept for about 30 seconds to 1 minutes and rinse with water.
4. Iodine was placed for 1 minute and wash with water.
5. Then wash with 95% alcohol or acetone for about 10-20 seconds and rinse with water.
6. Safranin was added for about 1 minute and wash with water

8. Wash slide in a gentile and indirect stream of tap water.

9- Dry the slide in the air, and observe under a microscope.

2-10-1 -2 Spore formation

A drop of distilled water was placed on a clean glass slide. part of the pure colony floated on the center of nutrient agar for 7 days was, spreaded on the surface of the slide and left to dry and then fixed by

flame for 5 min. Ability of bacteria to form blackboards was tested by dyeing malachite green dye in the Schaeffer-Fulton method. The slide was washed slowly using sterile water, covered with safranin dye for 30 seconds and slide after that were washed with fresh distilled water and dried by heat, and examined by optical microscope (Pommerville, 2007).

2-10-1-3 Oxidase reagent

Three drops of the oxidase reagent prepared in paragraph (2-3-1) were placed on a dry and clean filtration sheet, then a bacterial colony at the age of (18-24) hours was transported by sterile wooden stick, where the color of the colonies in dark violet after the passage (20-10) sec is given the positive test result (Tang & Stratton, 2006).

2-10-1-4 Catalase test

This test used to investigation the abililty of bacteriato produce catalase enzyme by placing part of the bacterial colony at the age of (18-24) hours into a clean glass slide by the carrier (loop) .A drop of dcatalase reagent was added as a hydrogen master with a concentration of 3%, the appearance of bubbles evidence of the positive result (Tang & Stratton, 2006).

2-10-1 -5 Motility test

The bacteria were cultured on motility media by stabbing incubated at 37 °C for 24-48 hours. The growth of bacteria in the middle around the stab range is evidence of the of postive result bacteria to movement.

2-10- 1-6 Indole test

The test used to detected the production of tryptophane free ring tubes containing the indole medium prepared in the paragraph (2-2-1-

6) were inoculated with bacterial culture1-3 colonies, at the age of 18-24 hour and incubated at 37°C for 48 hours and 2-5 drops of Kovac's dreagent were added with good shaking. The appearance of red ring indicated a positive result (MacFaddin, 2000).

2-10-1-7 sugar fermentation test

The tubes contain of sugar fermentation media to be tested (maltose, mannose, maltose, glucose, cellulose) prepared in the in the paragraph (2-2-1-8) were inoculated with bacterial culture colonies, at the age of (18-24) hour and incubated at 28°C for 7days The change color to yellow indicated a positive result (MacFaddin, 2000).

2-11 Api Systems (bioMerieux)

Api 20E and Api staph are identification system for Enteriobacteriaceae and staphylococcus respectively. Each system contains 20 standardized and miniaturized biochemical tests and database. These Api systems include a strip consisting of 20 microtubes containing dehydrated substrates. These tubes are inoculated with a bacterial suspension that re-constitutes the media.During incubation, metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the reading table and the identification is obtained by referring to the analytical profile index.

2-12 DNA was extracted using Kit

1- One ml of cells was placed in Eppendorf tube (OD 600 0.8-1.0) by centrifuging at 13,000 rpm for 1 min. After that, removed supernatant.

After centrifugation removed the supernatant and completely resuspend by vortex or tapping.

2- Fifty µl of pre- Buffer was added and 3 µl of lysozyme, mixed well.

3- Incubate at 65 °C for 15 min. To help lyse cells, invert mixed with the tube every 5 min during the incubation.

4- Tow hundred and fifty µl of G- Buffer solution, and invert mixed well

5- Incubate at 65 °C for 15 min. To help lyse cells, in vortex mixed with the tube every 5 min during the incubation.

6- Tow hundred and fifty µl of binding buffer, and completely mixed well by pipetting (at least 10 times) or gently vortex.

7-Cell lyateswas loaded on column, and centrifuged at 13,000 rpm for 1min.

8-Five hundred µl of washing buffer A was added, to column and centrifuged for 1 min at 13,000.

9- Five hundred µl of washing buffer B, was added to column and centrifuged at 13,000 rpm for 1 min.

10- Solution removed and centrifuged for 1 min at 13,000 rpm.

11-Placed the G- spin column in a clean 1.5 micro centrifuge tube, and added 50-200 µl of elution buffer directly on to the membrane

12- Incubate at room temperature for 1 min centrifuging at 13,000

2-13 Checks of DNA concentration

Nanodrop® Quawell UV-V spectrophotometer Q 5000 at a wavelength 230 nm was applied to determine the DNA concentration (ng/µl) and protein with humic acids. Protein purity (A260/A280) and humic acid purity (A260/A280) were measured.

2-14 Polymerase Chain Reaction (PCR) for pure bacterial colonies

After DNA extractions, polymerase chain reaction (PCR) was performed to amplify the16S rRNA gene to confirm the identity of *B. subtilis* isolates. Each PCR tube mix contained the following components:

- Tweleve and ahalf µl of Taq polymerase.
- One µl from each 27F (10 pmol/µl) primer and reverse primer 1492R (10 pmol/µl).
- Three µl of DNA extraction as a template.
- Complete the solution to the final volume 25 µl with free nuclease water.

After that, PCR-mix tubes were closed and transferred into the thermocycler. Thermal cycling was conducted in a GeneAmp PCR System 080725 (Multigene, Labnet International Inc.), under the following conditions: initial denaturation at 95°C for 2 min; 30 cycles; denaturation at 95°C for 1 min; annealing at 56°C for 1 min; elongation at 72°C for 2 min; final extension at 72°C for 7 min.

2-15 Analysis of PCR products

PCR products were loaded onto 1 % agarose gel electrophoresis which was prepared according to (Shaio *et al*. 1977) as the following

1 -One gram of agarose was dissolved in 100 ml of TBE (pH 8.3).

2- The solution was heated (using water bath), and the solution was allowed to warm at 50-60 ºC and 4 µl of red -safe stain was added.

3- The agarose gel was assembled to casting tray and comb was positioned at one end of the tray after both edges were sealed and agarose was allowed to gel at room temperature.

4- The comb was carefully removed and the gel was placed in electrophoresis chamber. The chamber was filled with TBE buffer until the buffer reached 0.5-1 mm over the surface of the gel.

5 -PCR product was mixed with 1µl of 6× loading dye and was loaded in the wells of the agarose gel.

6- The cathode was connected to the well side of the unit and the anode to the opposite side.

7- The gel electrophoresis was run at 90v for 1h.

8- The DNA band was observed under U.V. transilluminator.

2-16 Sequence analysis of pure colonies

The PCR products were purified using a clean kit (Favorv PREP^{TM} PCR Clean–Up Mini (FAVORGEN Biotechnology CORP., KOREA) according to the manufacturer's instructions.

2-16-1General protocol Sequence analysis of pure colonies

1-Placed 10-100 µl of PCR product to micro-centerifuge tube, and added 5 volumes of FAPC buffer mixed and welledl by vortexing.

2-Placed a FAPC column into a collection tube.

3- The sample mixtured was transfered to the FAPC column, centrifuge at 11,000 rpm for 30 seconds, then discarded the flow–through.

4-Six hundred µl of wash buffer was added (ethanol added) to the FAPC column. Centrifuge at 11,000 rpm for 30 seconds, and then discarded the flow through.

5 -The centrifuge was repeated at full speed (18,000 rpm) for an additional 3 minutes to dry the column matrix.

6- Placed the FAPC column to an Elution tube (provided)

7- Fourty µl of elusion buffer or double distilled water (ddH_2O) was added to the membrane center of the FAPC column .Stand the FAPC column left for 1min.

8-Centrifugation at full speed (18,000 rpm) for 1min to elute DNA.

after that, the purified products and primers 27F and 1492R with concentration of 10 pmol were sent for sequencing at Macrogen laboratories (Macrogen, Inc., South Korea). Sequence results were then submitted to a BLAST search in GenBank (http:// blast.ncbi.nlm.nih.gov/Blast.cgi) to compare with the closest known alignment identities for the partial 16S rRNA sequences.

2-17 Haematological parameters

Seven fish were randomly selected from each group and switched to an anesthetic bath of clove oil and sedated. Blood was withdrawn from the caudal vein puncture using 3 ml syringes (G 23) represented in figure (2-4) . Blood was placed in separate 1.5 ml EDTA tubes, to determine the haemoglobin (HB), red blood cells (RBC), white blood cells count (WBC) and hematocrit values (HCT) and differential leucocyte.

In order to separate serum a further amount of blood was transferred without the anticoagulant solution to separate 1.5 ml tubes and the blood was allowed to coagulate at room temperature for a while before centrifuging at 13,000 rpm. Blood serum was used to assess blood urea, cholesterol, serum creatinine, and random blood sugar (RBS) levels. The collected blood was moved to 1.5 ml eppendorf tubes and stored in refrigerator for further analysis. All above mentioned blood parameters were measured by autolysis machine (Swelab Alfa, Germany). Blood

serum was used also to assess the levels of selected cytokines(Al-Hisnawi *et al*., 2015)

Figure (2.4) illustrating collection of blood samples (3 ml) from the caudal vessel of *Cyprinus carpio*

2-18 Haematological indices

Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated from RBC, Hct and Hb according to the following formulae mentioned by Klontz (1997).

$$MCV = \frac{\text{Hct \%} \times 10}{\text{RBC mm3}}$$

$$MCH = \frac{\text{Hb} \times 10}{\text{RBC}}$$

$$MCHC = \frac{\text{Hb} \times 100}{\text{Hct}}$$

2-19 Immune tests by Enzyme Linked Immuno Sorbent Assay (ELISA)

To detect the level of Interleukin 1 β (IL1β) and TGF-β in serum samples, commercially available fish ELISA development kits (Elabscience®) were used.

2-19-1 Interleukin 1 β

In order to detect the level of Interleukin 1 Beta (IL1β) in serum samples, commercially available fish ELISA development kit was used.

Reagent preparation:

-All reagentswas brought to room temperature before use.

Standard: reconstitute the 120 µl of standard (80 ng/l) with 120 µl of standard dilution to generate a 40 ng/l standard stock solutn. Allow the standard to sit for 15 min with gentle agitation prior to making dilution. Prepare duplicate standard stock points by serially diluting the standard stock solution (40 ng/l) 1:2 with standard dilution to produce 20 ng/l, 10 ng/l, 5 ng/l, and 2.5 ng/l solution; standard diluent serves as the zero standard (0 ng/l). Any remaining solution should be frozen at -20 °C and used within one month.

1- All reagents was prepared, standard solution and sample as instructed brought all reagents to room temperature before use. The assay is performed at room temperature.

2- The number of strips was determined required for the assay. And inserted the strips in the frames for use.

3- Fifty µl of standard was added to standard well without adding antibody to standard well because the standard solution contains biotinylated antibody.

4- Fourty µl of sample was added to sample wells and add 10 µl anti–IL-1β antibody to the sample wells. Then add 50 µl streptavidin- HRP to the sample wells,and standard wells mixed well. Covered the plate with a sealer. Incubated for 60 min at 37 °C.

5-Removed the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml washed buffer 30 seconds to 1 min for each wash. For automated washing, aspirate all wells and wash 5 times with

washed buffer, overfilling wells with wash buffer. Blot the plate onto paper towels.

6-Fifty µl of substrate solution A was added to each well and then added 50 µl substrate solution B to each well. Incubate plate covered with new sealer for 10 min at 37 °C in the dark.

7- Fifty µl of stop solution was added to each well, the blue color will change in to yellow immediately.

8-Determine the optical density (OD value) of each well immediately using a micro plate reader set to 450 nm within 10 min after adding the stop solution.

2-19- 2 Transforming growth factor beta (TGF-β)

Reagent preparation

-All reagents was brought to room temperature before use. Standard: reconstitute the 120 µl of standard (4800 ng/l) with 120 µl of standard dilution to generate a 2400 ng/l standard stock solution. Allow the standard to sit for 15 min with gentle agitation prior to making dilution. Prepare duplicate standard stock points by serially diluting the standard stock solution (2400 ng/l) with standard dilution to produce 1200 ng/l, 600 ng/l, 300 ng/l, and 150 ng/l solution standard diluent serves as the zero standard (0 ng/l). Any remaining solution should be frozen at -20 °C and used within one month.

1- All reagents was prepared ,standard solution and samples as instructed. brought all reagents to room temperature before use. The assay is performed at room temperature.

2- The number of strips was determined required for the assay. And inserted the strips in the frames for use.

3- Fifty µl of standard was added to standard well without added antibody to standard well because the standard solution contains biotinylated antibody

4- Fourty µl of sample was added to sample wells and 10µl of anti-TGF-β antibody added to the sample wells. Then 50µl of streptavidin- HRP added to the sample wells and standard wells mixed well. Covered the plate with a sealer. Incubated for 60 min at 37 °C.

5-Removed the sealer and wash the plate at 5 times with washed buffer. Soak wells with at least 0.35 ml wash buffer 30 seconds to 1 min for each wash. For automated washing, aspirate all wells and washed 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels.

6- Fifty µl of substrate solution A was added to each well and then 50µl of substrate solution B added to each well. Incubated plate covered with new sealer for 10 min at 37 °C in the dark.

7- Fifty µl of stop solution was added to each well, the blue color will change in to yellow immediately.

8-Determined the optical density (OD value) of each well immediately using a micro plate reader set to 450 nm within 10 min after adding the stop solution.

2-20 Growth parameters

Upon termination of the feeding trials, growth performance parameters including percentage weight gain (WG), specific growth rate (SGR; per cent increase in body weight day^{-1}) feed conversion ratio

(FCR) and feed conversion efficiency (FCE) were measured following these formulae:

$$\text{FCE} = B/FI \times 100$$

$$\text{SGR} = \ln FW - \ln IW/t \times 100$$

$$\text{FCR} = FI/FW - IW$$

$$\text{DWG} = FW - IW$$

Where IW is the initial weight (g), FW is the final weight (g), FI is feed intake (g), t is the duration of feeding (in days), B = biomass (g) and Ln = natural logarithm value.

2-3 Statistical analyses

All results are presented as mean ± SD of the replicates. An independent two- samples t-test was used to determine the haemato-immunological and growth performance parameters between the control and experimental groups. The statistically significant differences between microbial community (TVC and LAB) among gut regions were tested by use of one-way ANOVA followed by Duncan's multiple range tests. Two-way ANOVA was used to compare between culturable bacteria levels among diets, regions and interactions. All statistics were carried out using MiniTab statistical software version 16 (IBM, Pennsylvania, USA). The accepted levels of significance in all cases were $P < 0.05$.

Chapter three

Results and Discussion

3-Results

3-1 -Total viable count

The total viable count (TVC) associated with the intestine regions of common carp fed the experimental diets are presented in Figure 3-1

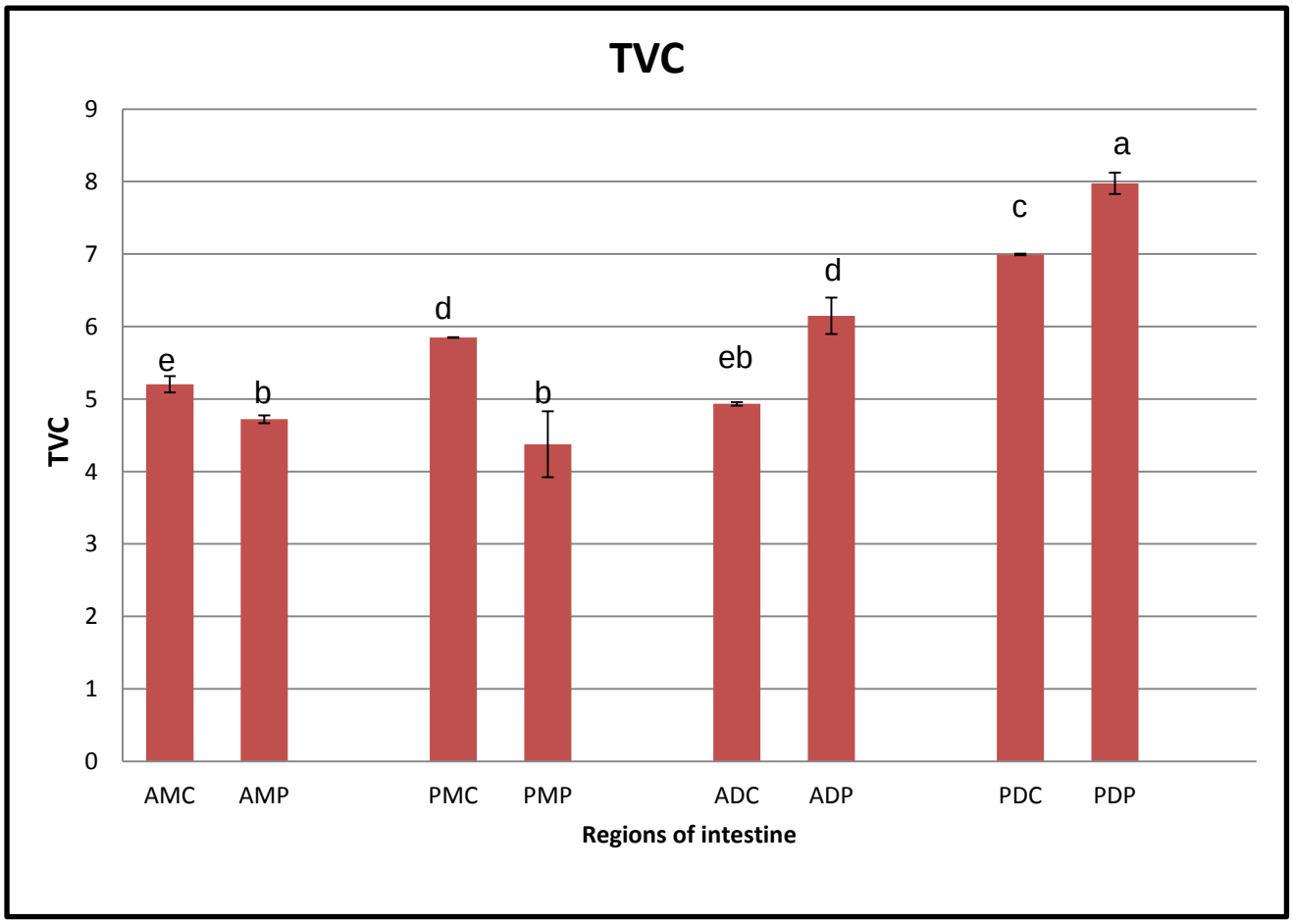


Figure (3.1) show the mean log of autochthonous (in the AM and PM) and allochthonous (in the AD and PD) TVC in the anterior and posterior intestine of common carp fed the probiotic or control diet at the end of the study

Bars with the different letters among regions are significantly different ($P < 0.05$). Values area mean ± SD represented by vertical bars of three replicatesGastrointestinal regions:AM anterior intestinal mucosa; PM, posterior intestinal mucosa; AD, anterior digesta; PD, posterior digesta; C, control; P, probiotic

Table (3-1) shows the two-way ANOVA analysisof diets,regions and interactions gastrointestinal regons

Two-way ANOVA						
	P value		Diets		Intestine regions	
Diets	Intestine regions	Interaction	Control	Probiotics	All regions	All regions
0.541	0.000	0.000	a	a	b	a

CultivableTVC levels in the control fed fish at the end of the experiment were log 5.2 ± 0.2, 5.8 ± 0.02, 4.9 ± 0.02 and 6.9 ± 0.009 CFU g^{-1} in the AM, PM, AD and PD, respectively. On the other hand, the TVC levels in the probiotic fed fish were log 4.7 ± 0.05, 4.3 ± 0.4, 6.1 ± 0.3 and 7.9 ± 0.1 CFU g^{-1}, respectively.

One-way ANOVA revealed that the number of TVC was significantly lower in the intestinal mucosa (AM and PM) of fish fed probiotic supplemented diet compared to the control group of fish. In contrast, intestinal digesta (AD and PD) revealed low significant numbers of the TVC in the control fed fish compared to the probiotic fed fish (P<0.05).

However, levels of cultivable TVC in the PD of the probiotic fed fish were found to be significantly higher compared to both regions and treatments (P < 0.001), while the intestinal mucosa (AM and PM) of the probiotic fed fish had significantly lower TVC levels in comparison to both intestine regions and treatments (P < 0.001). Finally, two-way ANOVA revealed significant differences between the TVC levels in respect to regions and interaction were recorded and no significant differences were found (P > 0.05) between the diets.

In the study of (Merrifield *et al*. 2010a) significant differences in the levels of total bacteria of rainbow trout after feeding *B. subtilis* were not found in comparison with the control group of fish. High levels of TVC (10.16 log CFU ml^{-1}) were reported in the intestine of common carp which were higher than the results of present study (Kaktcham *et al*., 2017). Furthermore, (Uddin and Al-Harbi, 2012) stated that the TVC (10-12 log CFU ml^{-1}) were recorded in the intestine of common carp. In the study of

(Hagi *et al.*, 2004) high levels of TVC were reported (equal to 1.9×10^9 in July and 7.2×10^7 in December), while the levels of LAB were 3.3×10^5 in July and 8×10^5 in December in common carp. These findings are similar to the LAB counts obtained from the present study. Autochthonous cultivable LAB in the present study were significantly decreased in *B. subtilis* fed fish compared to the control group. In the mucosa region.

Standen *et al*., (2015) reported that the feed containing *Lactobacillus reuteri*, *B. subtilis*, *Enterococcus faecium* and *Pediococcus acidilactici* given to tilapia significantly increased the levels of autochthonous LAB compared to the control group.

The differences in above mentioned studies could be attributed to such factors including differences in sampling and microbiological techniques and some factors related to fish (age and health status). The microbial community in the present study was comprised of common bacterial phyla described in fish, such as Firmicutes and Proteobacteria. Unfortunately, *B. subtilis* were not detected in the present study in either treatment. It could be molecular techniques including PCR-cloning, FISH and fluorescence microscopy are useful in identifying these bacteria. Indeed, *Bacillus* spp. have been identified as minor components of the microbial community in the common carp gut microbiota (Meng *et al.*, 2018; Allameh *et al.*, 2014).

The high prevalence of *Enterobacter* sp., *E. coli*, *Streptococcus* sp., and *Staphylococcus* sp. in this study suggests that these bacteria may be common in the intestine of carp. Further researches are needed using culture-independent techniques including (PCR-cloning and PCR-DGGE) to diagnose the microbial community of common carp in Iraq.

3-2 Lactic acid bacteria (LAB)

The lactic acid bacteria (LAB) associated with the intestine regions of common carp which was fed the experimental diets are presented in Figure3.2

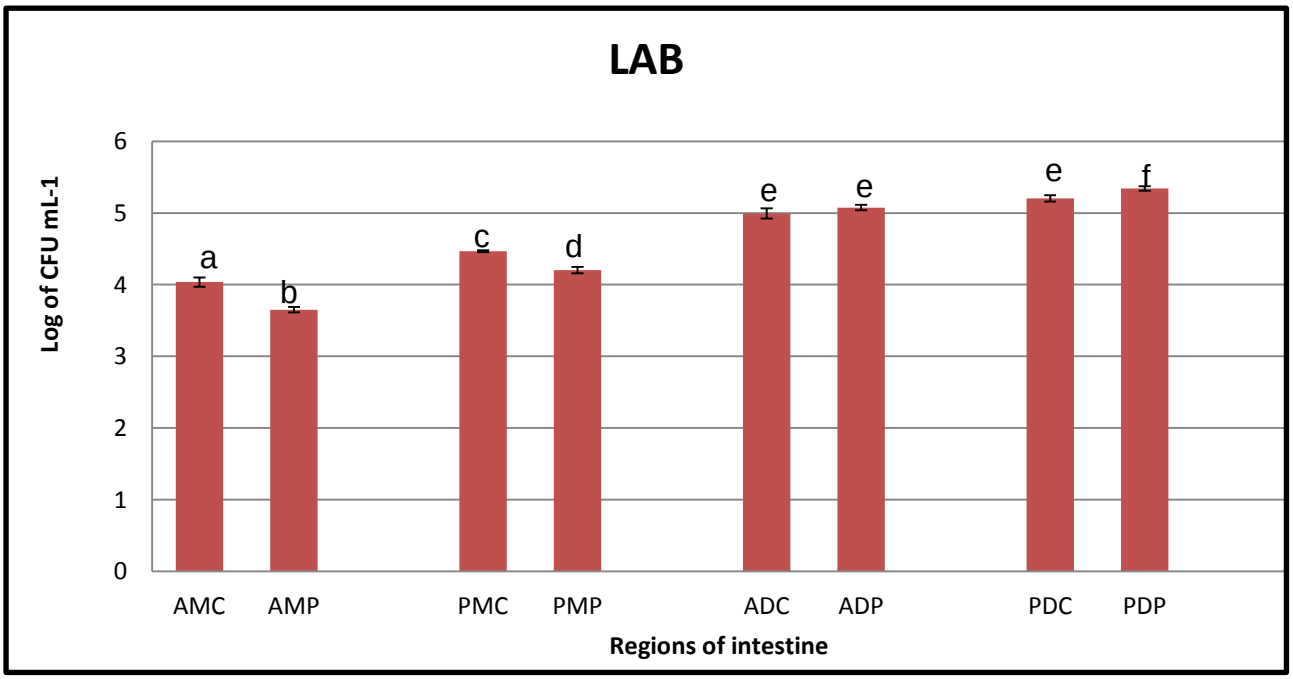


Figure (3. 2) shows the mean log of autochthonous (in the AM and PM) and allochthonous (in the AD and PD), LAB in the anterior and posterior intestine of common carp fed the probiotic or control diet at the end of the study

Bars with the different letters among regions are significantly different ($P < 0.05$). Values aremean ± SD represented by vertical bars of three replicates.. Gastrointestinal regions: AM, anterior intestinal mucosa; PM, posterior intestinal mucosa; AD, anterior digesta; PD, posterior digesta; C, control; P, probiotic

Table(3-2) shows the two-way ANOVA analysis of diets, regions and interactions

Two way –ANOVA						
	Pvalue		Diets		Intestinal regions	
Diets	Regions	Interaction	Control	probiotics	Regions	Regions
0.001	0.000	0.000	a	b	a	b

Cultivable LAB levels in the control fed fish at the end of the experiment were log 4.0 ± 0.1, 4.5 ± 0.01, 5.0 ± 0.1 and 5.2 ± 0.04 CFU g^{-1} in the AM, PM, AD and PD,

respectively. On the other hand, the LAB levels in the probiotic fed fish were log 3.6 ± 0.04, 4.2 ± 0.04, 5.1 ± 0.04 and 5.3 ± 0.03 CFU g^1, respectively

Lactic acid bacteria levels were significantly lower in in the intestinal mucosa (AM and PM) of common carp fed probiotic supplemented diet compared to the control group of fish (P = 0.001). Additionally, LAB levels were significantly higher in the PD of common carp fed probiotic diet compared with fish fed control diet (P < 0.05).

One-way ANOVA showed that, levels of LAB in the digesta samples (both AD and PD) were found to be significantly higher compared with mucosa samples (both AM and PM) (P < 0.001).

No differences were observed between ADC and ADP. In general, LAB levels were affected by diets (P = 0.001), regions (P = 0.000), and an interactive effect (P = 0.000) was observed Figure 3.2.

3-3 Microbial diagnosis

Approximately 40 colonies were selected and purified to give pure colonies. However, some of these isolates failed in purification in spite of several attempts that were conducted. The obtained colonies were then subjected to biochemical tests and Api20 to diagnosis to genus and species if possible presented in Table(3-3).

Table (3.3) Biochemical tests of bacterial isolates

Bacterial isolate	Gram stain	Indole	Catalase	Shape	Oxidase	Spore formation test	Motility test
Staphylococcus spp.	+	_	+	Cocci	-	-	-
Enterococcus spp.	+	+	-	Cocci	-	-	-
Enterobacter spp.	-	_	+	Rods	-	-	+
E. coli	-	+	+	Rods	-	-	-
Streptococcus spp.	+	+	-	Cocci	-	-	-

Lactococcus spp.	+	_	-	Cocci	-	-	-
Proteus spp.	-	+	+	Rods	-	-	+
Pseudomonas	-	_	+	Rods	+	-	+
Bacillus subtilus	+	-	+	Rods	-	+	+
Klebsiella pneumoiae	-	-	-	Rods	-	-	-

3-4 Microbial analysis

Due to such factors including their ability of spore formation and high resistance to physical and chemical conditions, *Bacillus* spp. are widely used as probiotics. Compared to the control group of fish, *B. subtilis* significantly reduced the total bacterial levels in the anterior and posterior mucosa and significantly increased TVC in the anterior and posterior digesta. The high bacterial levels in the intestine regions obtained from the present study may be due to optimum temperature (22 °C) for many mesophilic microbes in natural systems (Al-Harbi & Uddin, 2004).

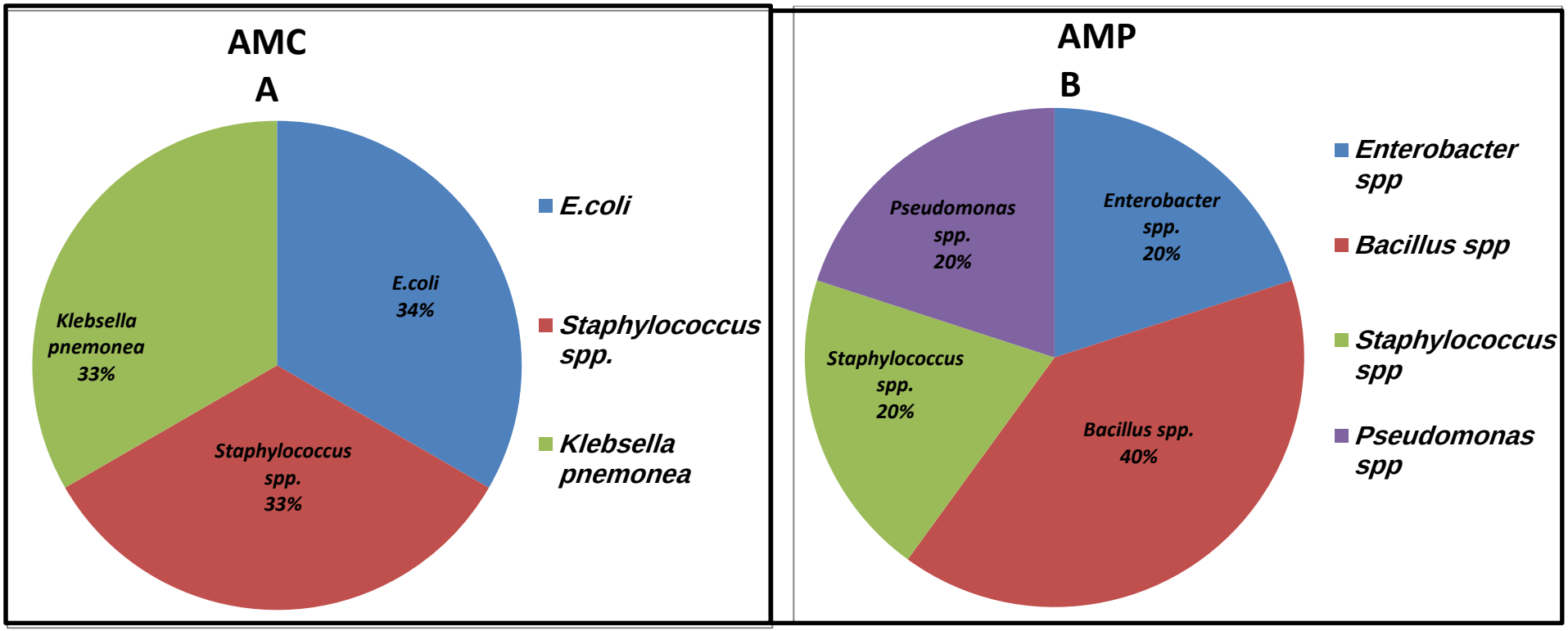
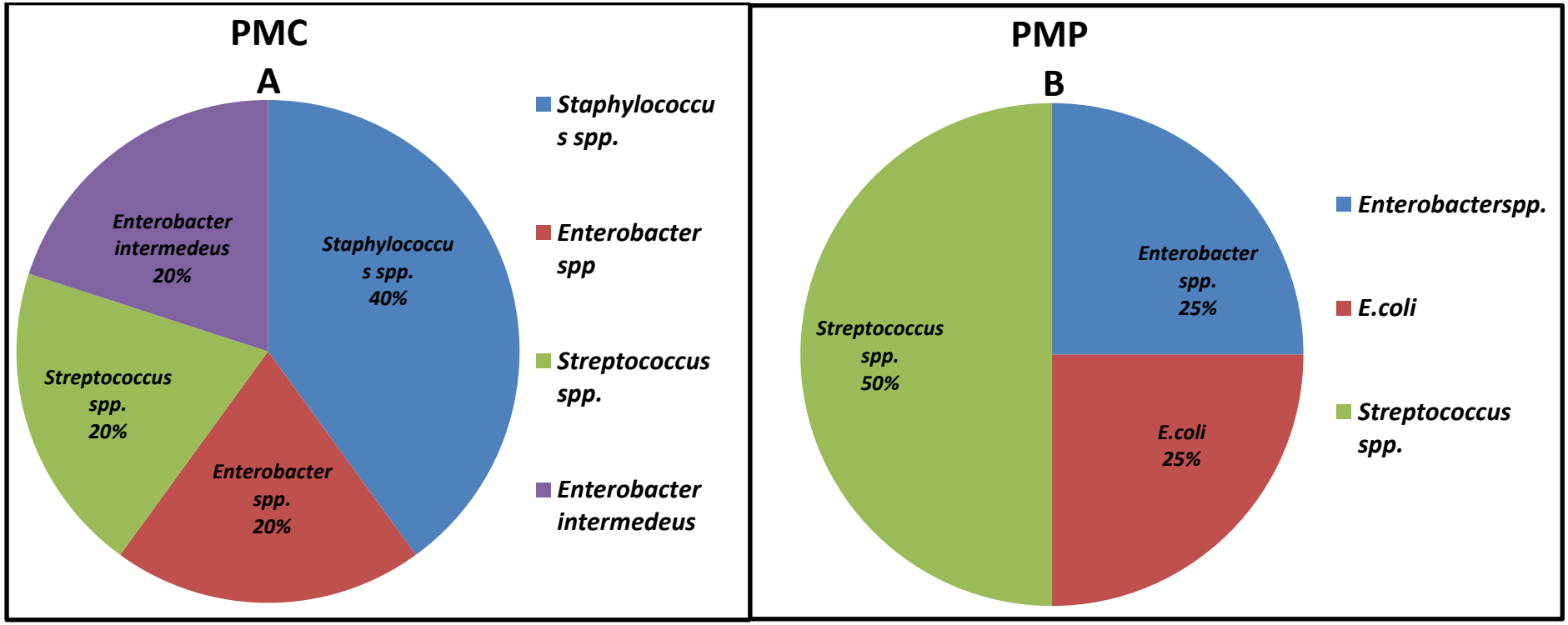


Figure (3. 3)Distribution of bacterial isolates in the in anterior mucosa common carp (A) anterior mucosa control (B)anterior mucosa probiotic

The identified bacterial isolates from the AM in both groups of fish fed probiotic and control diets are presented in Figure (3-3). Three genera were identified as *E. coli* (34%) *Klebsiella pneumonia* (33%) and *Staphylococcus spp*(33%). in the AMC. The dominant intestinal bacteria in the AMP were identified as *Bacillus* spp(40%), *Enterobacter spp. Staphylococcus spp*.(20%) and *Pseudomonas spp.* (20%) . *Klebsiella pneumoniae* as fish pathogen completely disappeared.(20%) in the AM of common carp fed *B. subtilis*

Klebsiella pneumoniae were isolated from the AM of fish fed control diet. Interestingly, these bacteria were inhibited in the AM of common carp fed *B. subtilis* diet. *Klebsiella pneumoniae* were identified in previous studies as a fish pathogen (Oliveira *et al.*, 2014; Das *et al.*, 2018)



Figure(3. 4)Distribution of bacterial isolates in the posterior mucosa in common carp (A) posterior mucosa control (B) posterior mucosa probiotic

The identified bacterial isolates from the PM in both groups of fish fed probiotic and control diets are presented in Figure 3-4.

Four bacterial genera were detected in the PMC as *Staphylococcus spp*(40%) *Enterobacter spp*(20%), *Streptococcus spp.*(40%) and *Enterobacter intermedius* (20%), while *Streptococcus spp.*(50%) in PMP 50% *E. coli*(25%) and *Enterobacter spp.*(25%).

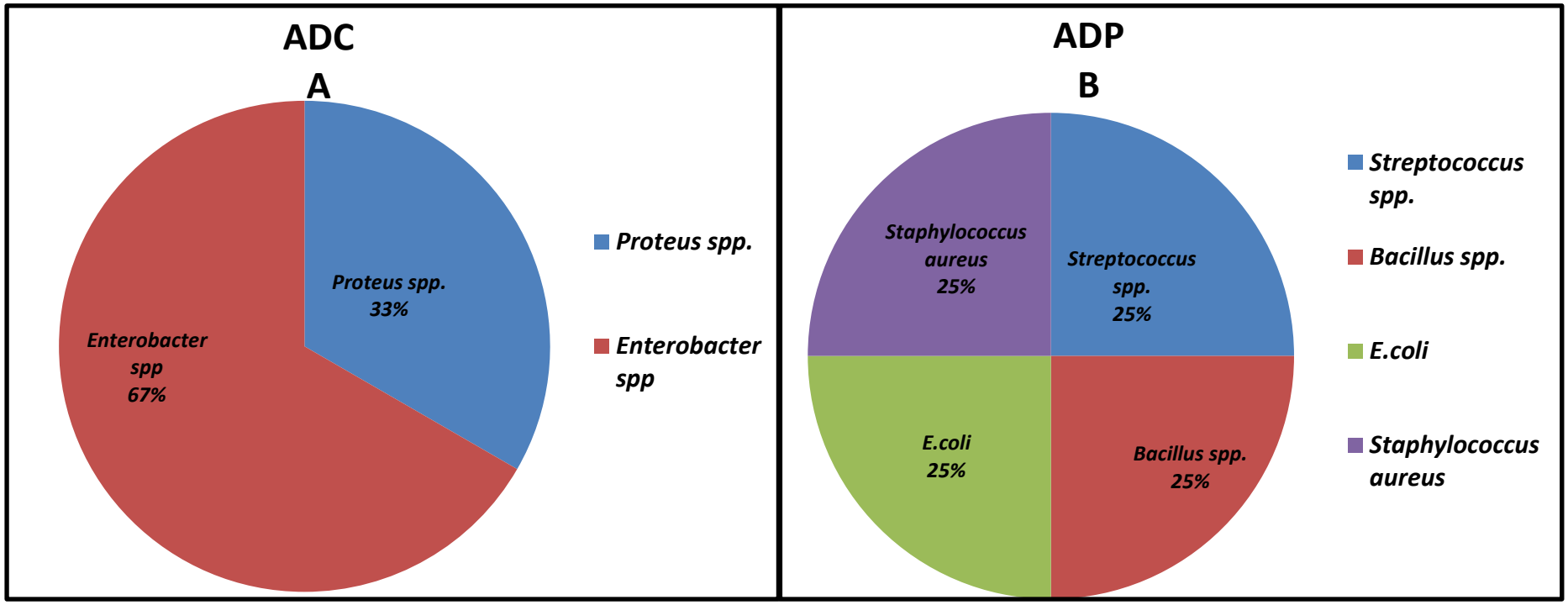


Figure (3. 5) Distribution of bacterial isolates in the anterior digestia in common carp (A) anterior digesta control (B) anterior digesta probiotic.

The identified bacterial isolates from the AD in both groups of fish fed probiotic and control diets are presented in Figure 3-5The genus was predominantly in ADC identified as *Enterobacter spp*,(67%). populations from these samples followed by *Proteus spp*.(33%,) whereas *Staphyllococcus aureus (25%) E. coli*,(25%) *Streptococcus spp*.(25%), and *Bacillus* spp. were detected in ADP

Staphylococcus spp., *Pseudomonas aeruginosa* and *Streptococcus* spp. are the predominant microbiota isolated from all regions in both treatments in the current study which have been widely identified from common carp in the previous studies (Kaktcham *et al*., 2017; Al-Harbi & Uddin, 2012; Dutilh *et al*., 2011; Uddin & Al-Harbi, 2012; Allameh *et al*., 2014).

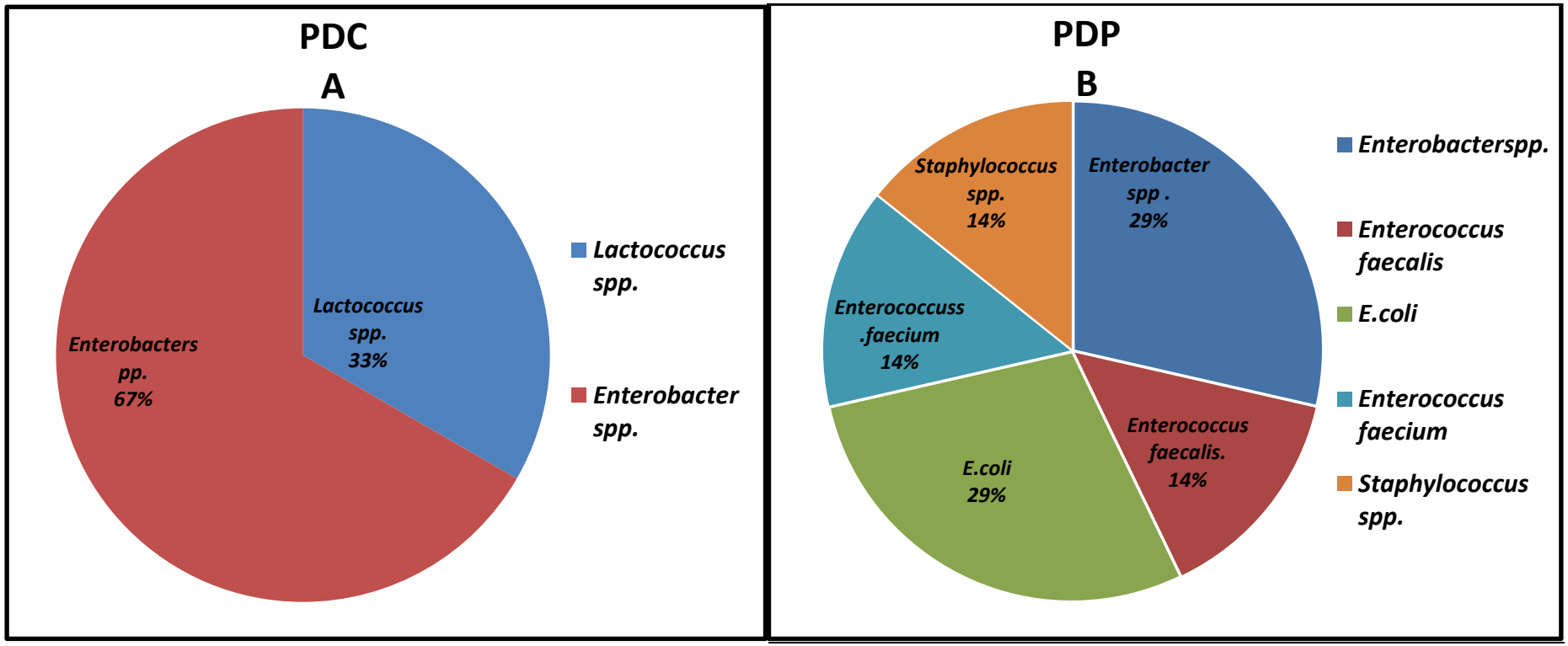


Figure (3. 6)Distribution of bacterial isolates in the posterior digestia in common carp (A) posterior digesta control (B) posterior digesta probiotic

The identified bacterial isolates from the PD in both groups of fish fed probiotic and control diets are summarized in Figure 3-6

Dominant bacterial genera from the PDC were identified as *Enterobacterspp*(67%)., and *Lactococcus spp*.(33%) whereas more diversity of bacterial genera were detected in PDP represented by *Enterobacter spp.* (29%) and *E. coli* (29%), followed by *Enterococcus faecium*,(14%) *Enterococcus faecalis,(14%)* and *Staphylococcus spp*.(14%) .

The high prevalence of *Enterobacter* sp., *E. coli*, *Streptococcus* sp., and *Staphylococcus* sp. in this study suggests that these bacteria may be common in the intestine of carp. Further researches are needed using culture-independent techniques including (PCR-cloning and PCR-DGGE) to diagnose the microbial community of common carp in Iraq.

The results of microbial diagnosis by biochemical tests indicated that the dominant microbes were found to be *Enterobacter* spp. in almost all regions. *Enterobacter* spp. isolates were also retrieved in the groups fed with the probiotics, in both digesta regions and anterior mucosa. Dominant bacterial genera from all feeding groups were identified as *Enterobacter* spp., followed by *Streptococcus* spp., *E. coli*, *Bacillus* spp. and *Staphylococcus* spp.

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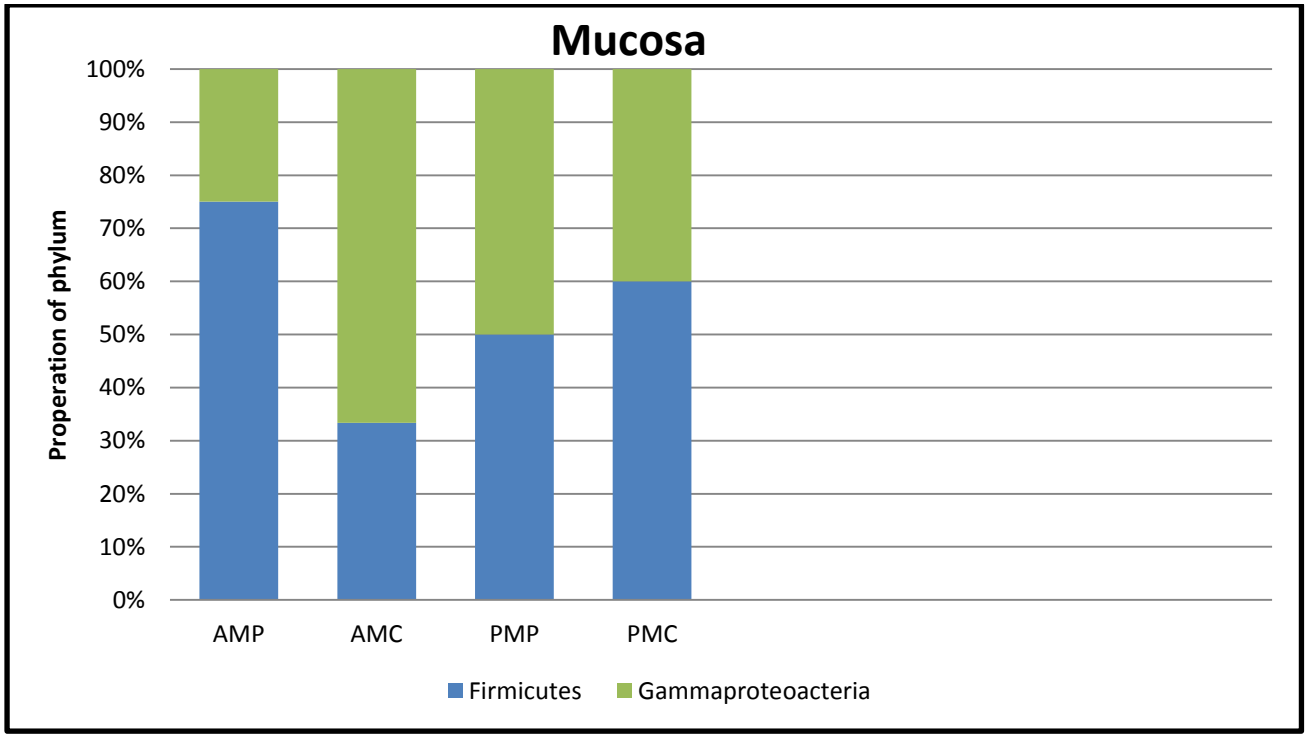

Figure (3- 7) The rerelative proportions of bacterial phyla within both mucosa regions from probiotic and control groups. Key words: AMC=Anterior mucosa control. AMP= Anterior mucosa probiotic. PMC= Posterior mucosa control . PMP= Posterior mucosa probiotic

Representative gut isolates obtained from diagnosis by biochemical tests were classified into two phyla: Gammaproteobacteria and Firmicutes in the mucosa and most digesta samples (Figures 7and8).

The relative proportions of Gammaproteobacteria were 66.7%, 40%, 40% and 50% in the AMC, AMP, PMC and PMP, respectively, while the relative proportions of Firmicutes were 33.3%, 60%, 60% and 50%, in the AMC, AMP, PMC and PMP, respectively (Figure 3-7).

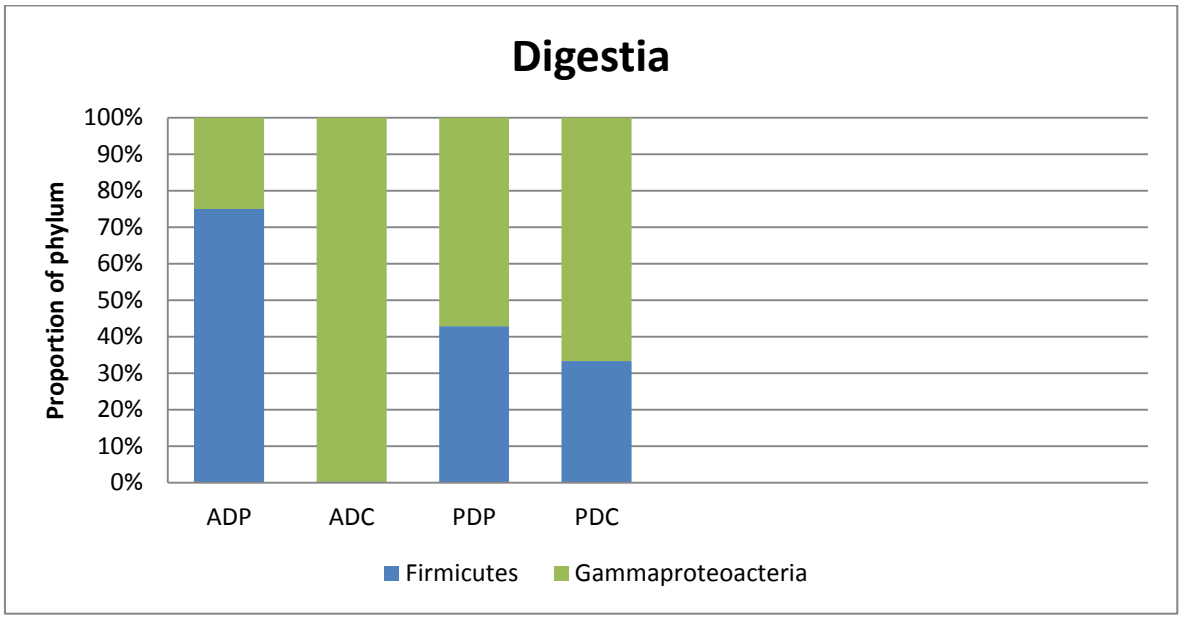

Figure (3- 8) The relative proportions of bacterial phyla within both digestia regions from probiotic and control groups . Key words: ADC=Anterior digestia control. ADP= Anterior digestia probiotic.PDC= Posterior digestia control . PDP= Posterior digestia probiotic

At the end of the present study, Gammaproteobacteria phyla was identified in the digesta samples which accounted for 100%, 25%, 66.7% and 57.1% in the ADC, ADP, PDC and PDP, respectively, followed by Firmicutes which accounted for 0%, 75%, 33.3% and 42.9% in the ADC, ADP, PDC and PDP, respectively (Figure 3-8).

Previous studies demonstrated that the intestinal microbiota of freshwater species tend to be dominated by genera of the phyla Proteobacteria and Firmicutes, among these bacteria Enterobacteriaceae, which have been commonly isolated from common carp (Kaktcham *et al*., 2017; Dutilh *et al*., 2011; Allameh *et al*., 2014). In accordance with aforementioned studies, the current study revealed that some species belong to Enterobacteriaceae (including *Enterobacter* spp., and *E. coli*) were identified in all gastrointestinal tract regions of common carp.

3-5 Haematology parameters

The effect of *B. subtilis* supplemented diet on common carp haematological profiles is presented in Table (3-4)

Table (3-4) Hematological parameters of common carp fed the control diet and the control diet supplemented with the probiotic *Bacillus subtilis*.

Blood parameters	Control	Probiotics	*P* value
Whiteblood cell ($\times 10^3 mm^3$)	35.4 ± 1.1^a	49.7 ± 8.3^a	0.059
Lymphocyte (%)	91.1 ± 9.7^a	95.8 ± 1.5^a	0.54
Granulocytes (%)	0.35 ± 0.2^a	1.2 ± 0.5^a	0.129
Monocytes, eosinophils, basophils and blasts. (%)	1.75 ± 0.9^a	3.05 ± 0.9^a	0.448
Erythrocyte count ($\times 10^6$ mm^3)	0.30 ± 0.2^a	0.25 ± 0.05^a	0.128
Hemoglubin (g dL^{-1})	9.6 ± 0.6^a	10.1 ± 1.5^a	0.136
Hematocrit	4.5 ± 0.9^a	7.4 ± 1.4^b	0.030
Mean corpuscular volume(fL $cell^{-1}$)	204.2 ± 1.4^a	216.6 ± 7.8^b	0.027
Mean corpuscular haemoglobin(pg $cell^{-1}$)	463.6 ± 1.4^a	350.9 ± 60.5^a	0.231
Corpuscular haemoglobin concentration(g dL^{-1})	161.3 ± 22.8^a	214.4 ± 10.0^b	0.024

Means with the same letter in each raw are not significantly different ($P > 0.05$). The data are expressed as the means ± S.D (n = 7). (MCV) mean corpuscular volume, (MCH) mean corpuscular haemoglobin, (MCHC) mean corpuscular haemoglobin concentration, (MID) monocytes, eosinophils, basophils and blasts.

During the current study, erythrocyte numbers, mean corpuscular haemoglobin content (MCH) and haemoglobin were not significantly ($P > 0.05$) affected by the dietary probiotic. However, mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV) haematocrit were significantly increased in the probiotic fed fish compared to the control group ($P<0.05$).

The WBC count showed a clear increase in the probiotic fed fish trend to be significant ($P=0.059$). In contrast, MID and granules count was increased in the fish

fed the supplemented probiotic diet but no significant differences were observed in comparison to the control fed fish.

It is commonly believed that the haematological and blood biochemistry are good indicators for monitoring health condition in fish following feeding on probiotic feed additives and different stresses in fish farming (Ahmadifar *et al*., 2019).

In addition, awareness of haematological characteristics is an important tool that has been successfully used to track physiological changes in fish as an accurate and sensitive index (Satheeshkumar *et al*., 2011). Normal ranges for various blood parameters in fish have been established by different investigators in fish physiology and pathology (Xiaoyun *et al*., 2009).

In the present study, haematocrit, MCV and MCHC were significantly higher in the probiotic fed fish group in comparison to the control group. Several studies, have reported that the contradictory data of haematology parameters were obtained after administration of *B. subtilis* in different species of fish. For example, RBC, haemoglobin content and WBC were decreased and improved, respectively in Indian major carp *Labeo rohita* fed diet supplemented with *B. subtilis* (Kumar *et al*., 2006). In a study (Kamgar *et al*. 2014) reported no improvement of haemtology parameters on addition of the *B. subtilis* in the nutrient rations of the rainbow trout. A previous study also reported increased number of RBC, WBC and Hb content in rainbow trout supplemented with LAB added diets (Faramarzi *et al*., 2011). Groups of common carp had improved levels in HCT, RBCs, WBCs, Hb when fed different bacterial strains as probiotics supplemented diets (Ahmadifar *et al*., 2019; Ahmadifar *et al*., 2020). The reason behind these inconsistent results is not clear but it could because of differences in probiotic species and levels, administrative time and variations in fish species. The above mentioned results indicate that *B. subtilis* leads to increase the percent of WBCs and lymphocyte which can stimulate the immune system in fish by this elevation (Ahmadifar *et al*., 2020).

Predominantly macrophages and neutrophils have shown direct effect on the health condition of fish by rising the innate immunity of the fish.Astudy revealed that the feeding of Gram-positive and Gram-negative probiotic bacteria in 10^7 cells g^{-1} of feed resulted in a substantial increase in the number of erythrocyte within two weeks of the feeding trial (Panigrahi *et al*., 2007); on the other hand, the increased number of white blood cell (WBC) aids in the immunity of non-specific neutrophils and macrophages.

Additionally, in some studies, heterogeneous expression of probiotic bacteria in Nile tilapia (*Oreochromis niloticus*) and other fish may increase the established levels of intraepithelial lymphocytes and acidophilic granulocytes, serum lysozyme activity and serum bactericidal activity according to fish specificity (Sharma *et al*. 2013).

However, higher counts (percentage) of phagocytic cells (neutrophils and monocytes) and lymphocytes suggest infection in fish. Probiotics communicate with immune cells including mononuclear phagocytic cells (monocytes and macrophages) and polymorphonuclear leucocytes (neutrophils) and NK cells in order to stimulate innate immune responses (Irianto and Austin, 2002a; Nikoskelainen *et al*., 2003; Kumar *et al*., 2008).

3-6 Serum biochemistry profiles

Serum biochemistry parameters of common carp fed the control diet and the control diet supplemented with the probiotic *Bacillus subtilis* are summarized in Table 3.5 Blood urea remained unaffected by the probiotic diet. The current data indicate that the serum creatinine value was significantly higer in probiotic fed fish compared to the control group of fish. In addition, fish fed dietary *B. subtilis* displayed higher serum cholesterol and random blood sugar levels compared to the control group, but no significant differences were observed

Table (3-5) Serum biochemical parameters of common carp fed with probiotic supplemented and control diets for 6 weeks

Biochemistry parameters mg dL-1	Control	Probiotic
Blood urea	$26 .2 \pm 1.8^{a}$	26.0 ± 3.9^{a}
Serum creatinine	0.8 ± 0.4^{a}	1.3 ± 0.6^{b}
Random blood sugar (RBS)	181.3 ± 62.4^{a}	188.5 ± 27.2^{a}
Blood cholesterol	124.0 ± 24.1^{a}	130.0 ± 13.6^{a}

Means with the same letter in each row are not significantly different ($P > 0.05$). The data are expressed as the means ± S.D (n = 7).

It can be useful to understand that the level of serum glycemia in fish is the fastest and most cost effective way to evaluate the stress condition (Silbergeld, 1974). Consequently, environmental stress may also cause a marked increase in serum glucose concentrations (Silbergeld, 1974). Blood urea nitrogen (BUN) was the result of protein catabolism urea concentrations are increased due to high protein diet or renal lesions. It is worth noting that serum Alanine aminotransferase (ALT) , creatinine and urea are considered essential criteria for the evaluation of unusual feed stuffs and new feed additives for their proper additional stage**.** Decreased levels of cholesterol indicate possible illness, increased levels of physiological discomfort (stress), and lipid metabolism dysfunction(Jacobson-Kram & Keller, 2001)..

The findings obtained from the current study revealed that blood urea serum creatinine (marker of the kidney function), random blood sugar (RBS) and blood cholesterol were not affected by adding *B. subtilis* to the feed of common carp for 6 weeks. In line with present results(Sharifuzzaman *et al*., 2014) demonstrated that serum haemoglobin, urea, creatinine and glucose of juvenile rainbow trout were not affected by feed supplemented with ~10^{7} cells g^{-1} *Rhodococcus* or ~10^{8} cells g^{-1} *Kocuria* preparation. Creatinine provides information on renal disease or post renal obstruction or leakage whereas increased blood creatinine concentration may reflect

renal dysfunction due to structural damage, while low concentration has no clinical significance (Jacobson-Kram & Keller 2001).

In contrast to the currents findings, (Panigrahi *et al.,* 2010) demonstrated that the commercial probiotics containing *Lactobacillus rhamnosus* significantly increased the plasma cholesterol of rainbow trout. An indication of good fish heath status increases alkaline phosphate production, erythrocyte sedimentation rate, total serum protein. Similarly,(Al-Dohail *et al.*, 2009) reported that total serum protein, Ca^{+2}, Mg^{+2}, Cl^{-}, glucose and cholesterol were significantly enhanced by the supplementation of *Lactobacillus acidophilus* in African Catfish diets. On the other hand,(Dawood *et al*., 2016) reported that *Lactobacillus rhamnosus* or/and *Lactococcus lactis* can decrease the plasma total cholesterol and triglyceride levels of red sea bream.(Naser *et al.,* 2019) found creatinine and urea levels in *Aeromonas hydrophila* infected fish decreased by dietary treatment of natural or intestinally isolated probiotic bacteria. Reduction of serum urea in probiotic groups could be due to their role in enhancing renal histology. Such findings have been associated with several researches that indicate creatinine and urea were heavily affected in fish infected by *A. hydrophila* after being fed by dietary treatment of *Bacillus spp.*

Species, diets fed or other environmental factors could be the reasons that resulted in the slight differences in above mentioned findings.(Panigrahi *et al*., 2010) stated that food-derived indigestible carbohydrate can be fermented by probiotic bacteria to produce short chain fatty acids in the gut which in turn inhibit hepatic cholesterol synthesis and/or redistributing cholesterol from plasma to the liver by decreasing the systemic levels of blood lipids. Moreover, some bacteria can decrease the cholesterol absorption levels from the gut with resulting effects on cholesterol metabolism or by directly assimilating cholesterol. The obtained results in the current study indicate that dietary probiotics had no adverse effects on the kidney and liver function.

A1-AF

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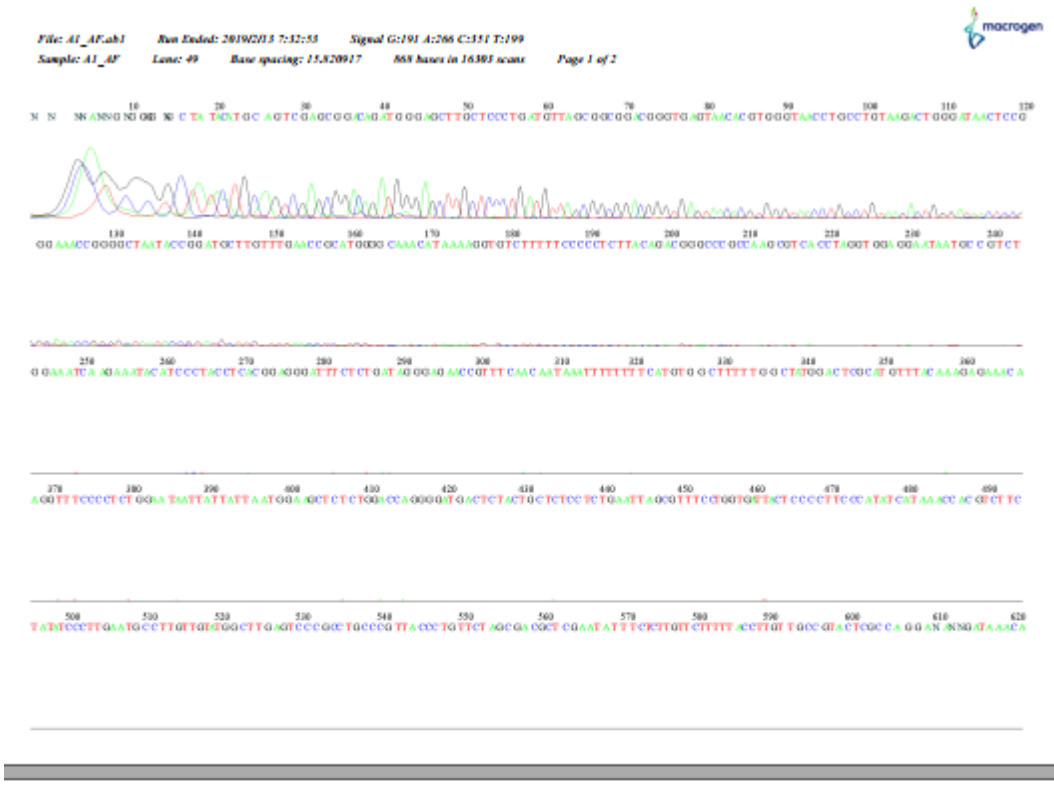
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Sample: A1_AF Lane: 49 Base spacing: 15.820917 868 bases in 16303 scans Page 1 of 2
macrogen


File: A1_AF.ab1 Run Ended: 2019/2/15 7:32:53 Signal G:191 A:266 C:351 T:199
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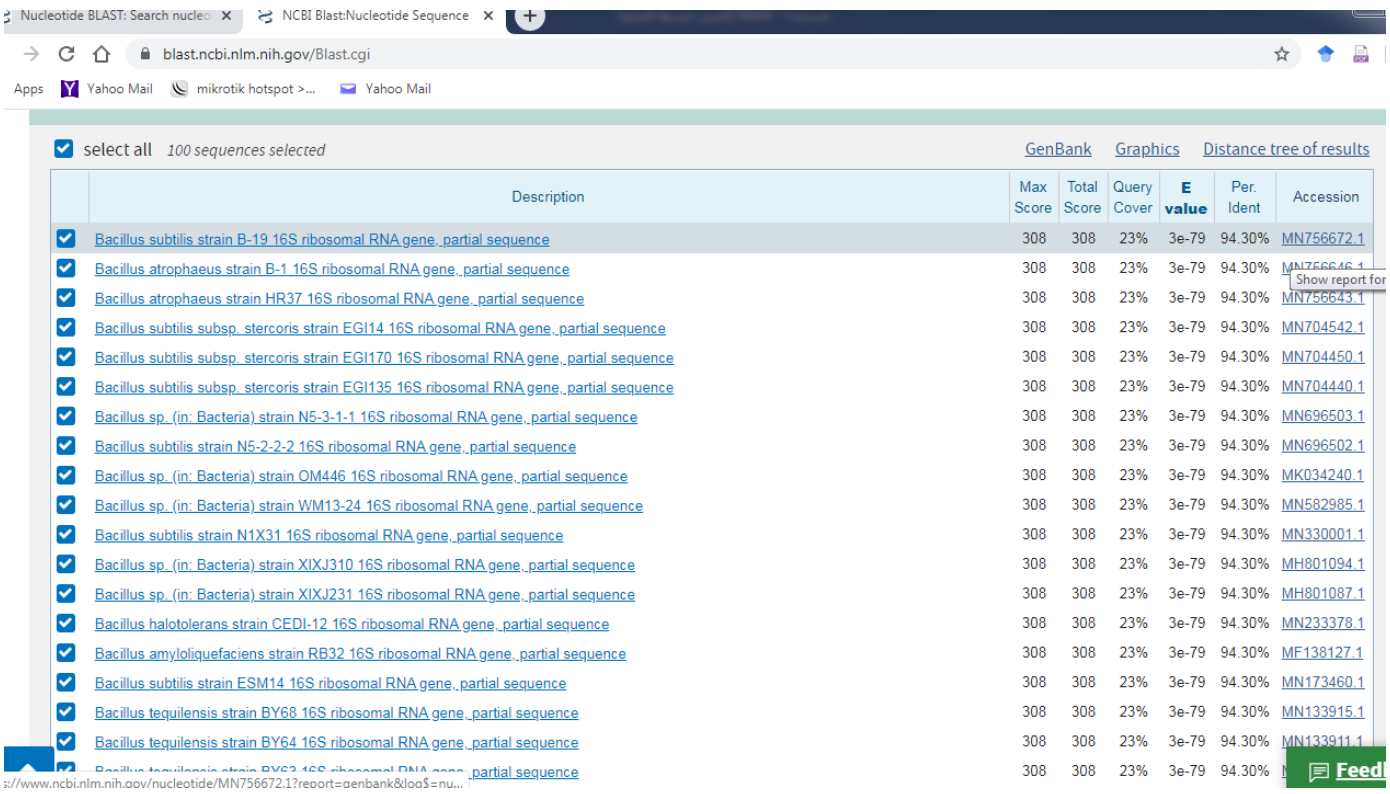

Figure(3-9) represented sequence results of *Bacillus subtilis* MN756672.1

A2-AF

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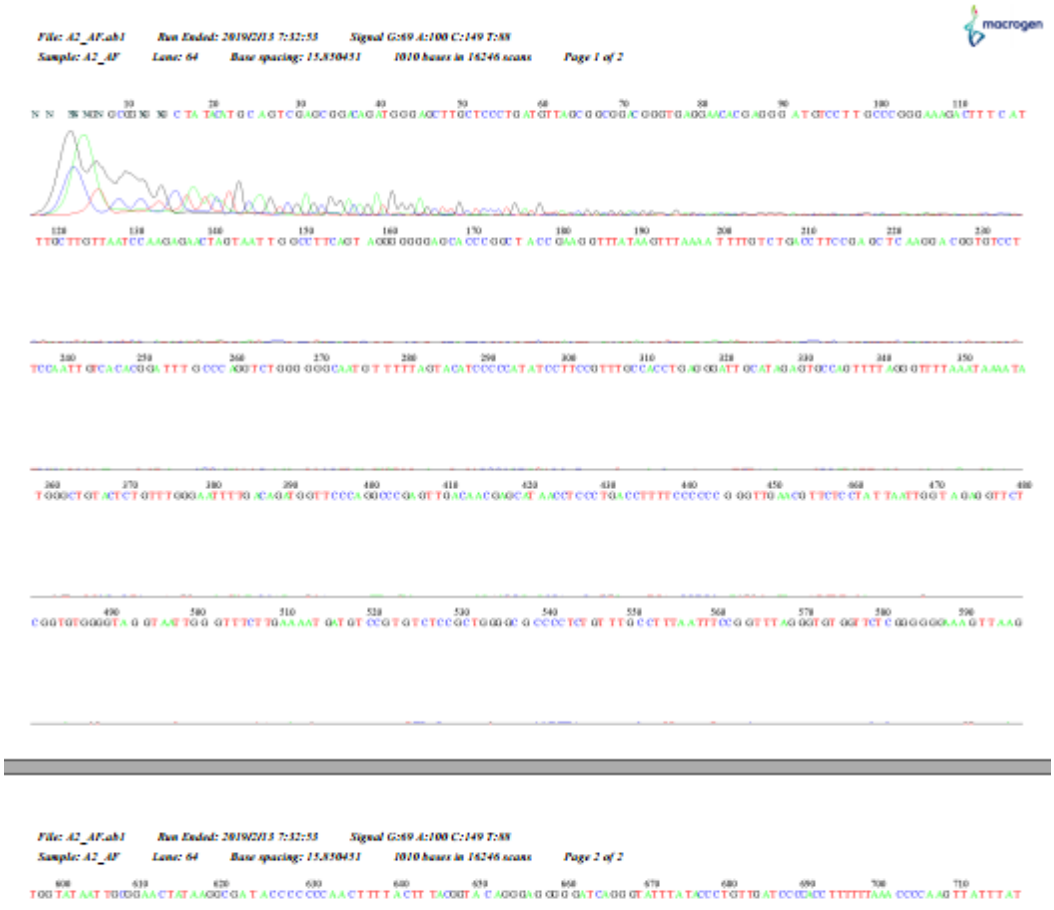

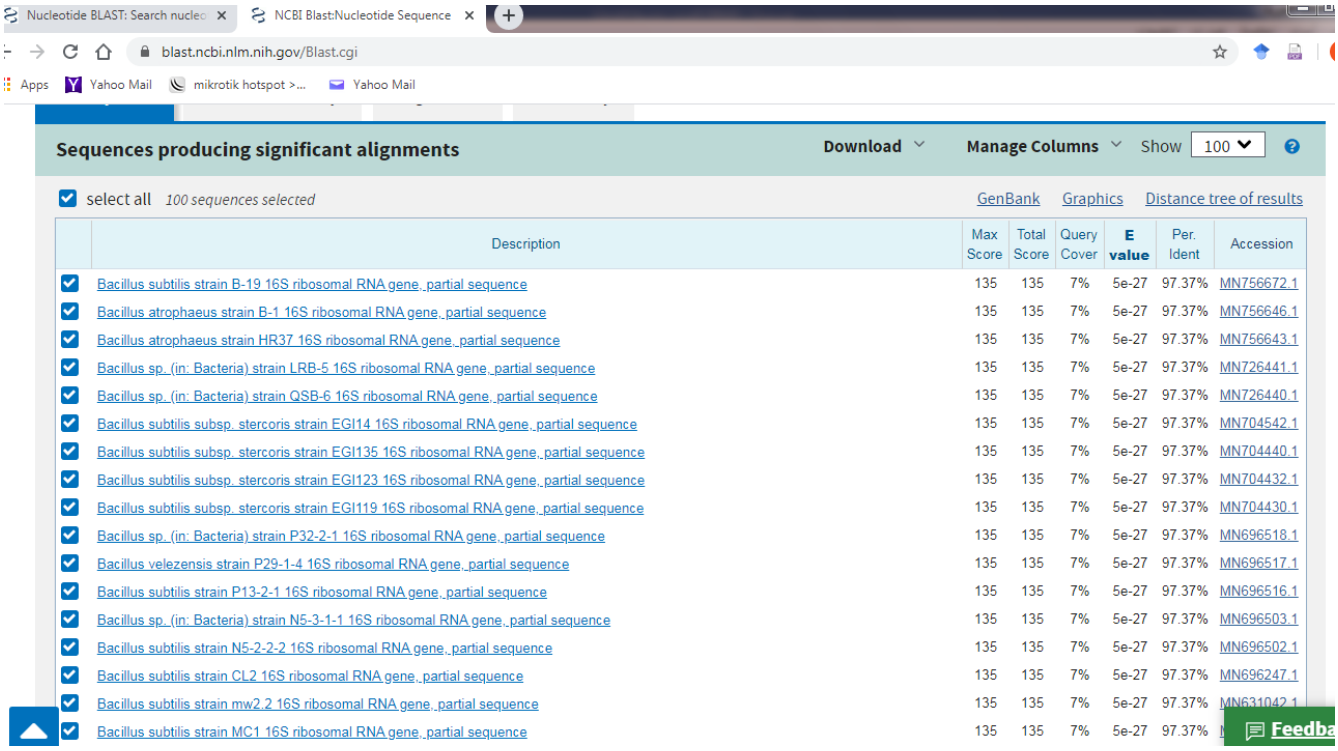

Figure(3-10) represented sequence results of *Bacillus atrophaeus* MN756672.1 .

A3AF

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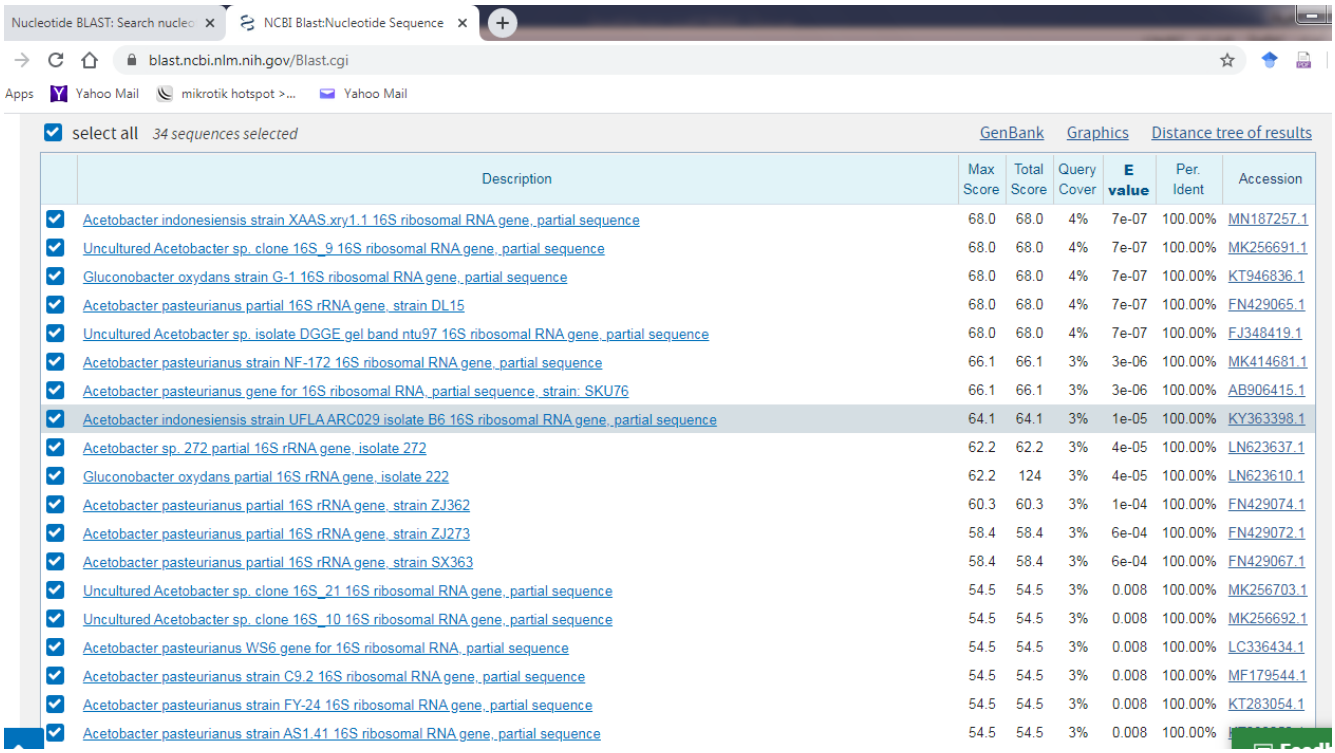

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Acetobacter indonesiensis strain XAAS.xry1.1 16S ribosomal RNA gene, partial sequence	68.0	68.0	4%	7e-07	100.00%	MN187257.1
Uncultured Acetobacter sp. clone 16S_9 16S ribosomal RNA gene, partial sequence	68.0	68.0	4%	7e-07	100.00%	MK256691.1
Gluconobacter oxydans strain G-1 16S ribosomal RNA gene, partial sequence	68.0	68.0	4%	7e-07	100.00%	KT946836.1
Acetobacter pasteurianus partial 16S rRNA gene, strain DL15	68.0	68.0	4%	7e-07	100.00%	FN429065.1
Uncultured Acetobacter sp. isolate DGGE gel band ntu97 16S ribosomal RNA gene, partial sequence	68.0	68.0	4%	7e-07	100.00%	FJ348419.1
Acetobacter pasteurianus strain NF-172 16S ribosomal RNA gene, partial sequence	66.1	66.1	3%	3e-06	100.00%	MK414681.1
Acetobacter pasteurianus gene for 16S ribosomal RNA, partial sequence, strain: SKU76	66.1	66.1	3%	3e-06	100.00%	AB906415.1
Acetobacter indonesiensis strain UFLA ARC029 isolate B6 16S ribosomal RNA gene, partial sequence	64.1	64.1	3%	1e-05	100.00%	KY363398.1
Acetobacter sp. 272 partial 16S rRNA gene, isolate 272	62.2	62.2	3%	4e-05	100.00%	LN623637.1
Gluconobacter oxydans partial 16S rRNA gene, isolate 222	62.2	124	3%	4e-05	100.00%	LN623610.1
Acetobacter pasteurianus partial 16S rRNA gene, strain ZJ362	60.3	60.3	3%	1e-04	100.00%	FN429074.1
Acetobacter pasteurianus partial 16S rRNA gene, strain ZJ273	58.4	58.4	3%	6e-04	100.00%	FN429072.1
Acetobacter pasteurianus partial 16S rRNA gene, strain SX363	58.4	58.4	3%	6e-04	100.00%	FN429067.1
Uncultured Acetobacter sp. clone 16S_21 16S ribosomal RNA gene, partial sequence	54.5	54.5	3%	0.008	100.00%	MK256703.1
Uncultured Acetobacter sp. clone 16S_10 16S ribosomal RNA gene, partial sequence	54.5	54.5	3%	0.008	100.00%	MK256692.1
Acetobacter pasteurianus WS6 gene for 16S ribosomal RNA, partial sequence	54.5	54.5	3%	0.008	100.00%	LC336434.1
Acetobacter pasteurianus strain C9.2 16S ribosomal RNA gene, partial sequence	54.5	54.5	3%	0.008	100.00%	MF179544.1
Acetobacter pasteurianus strain FY-24 16S ribosomal RNA gene, partial sequence	54.5	54.5	3%	0.008	100.00%	KT283054.1
Acetobacter pasteurianus strain AS1.41 16S ribosomal RNA gene, partial sequence	54.5	54.5	3%	0.008	100.00%	

. **Figure(3-11) represented sequence results of *Acetobacter indonesiensis* MN187257.1**

A4-AF

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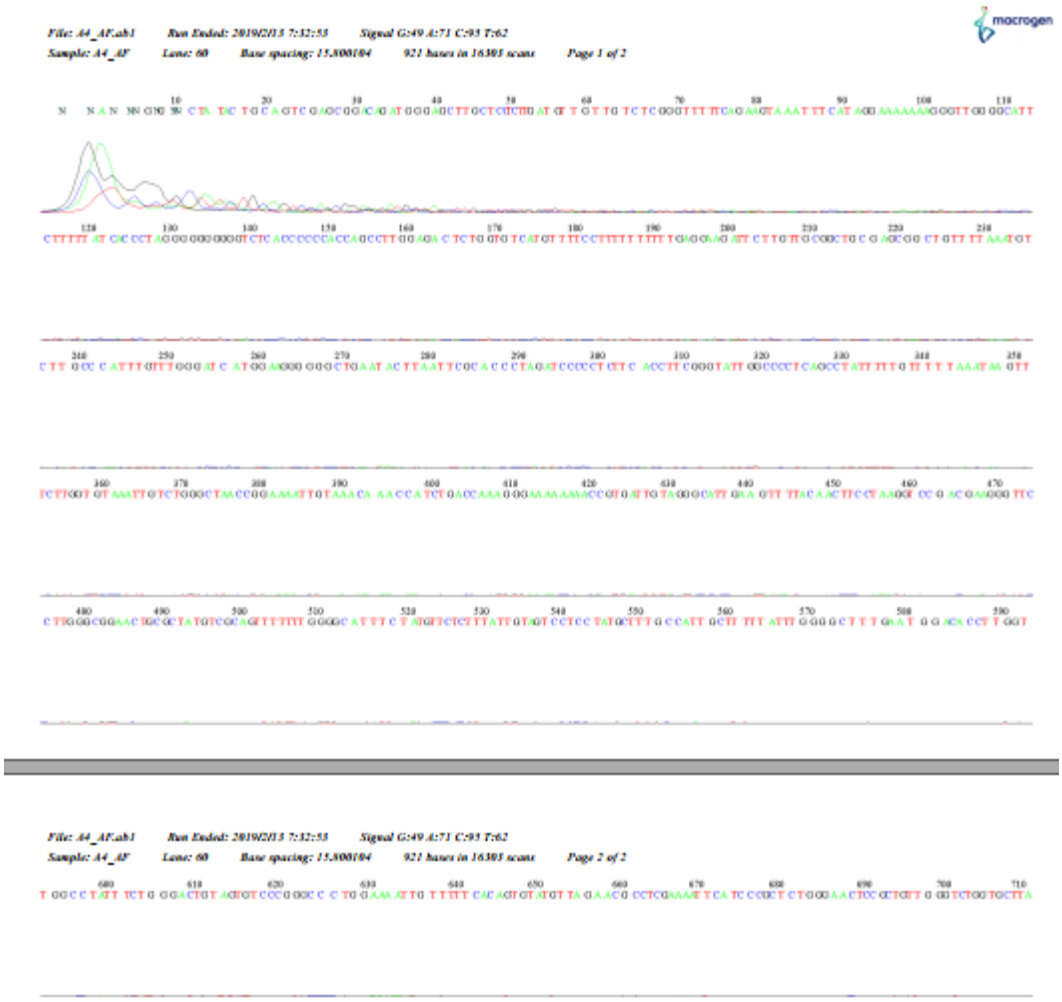

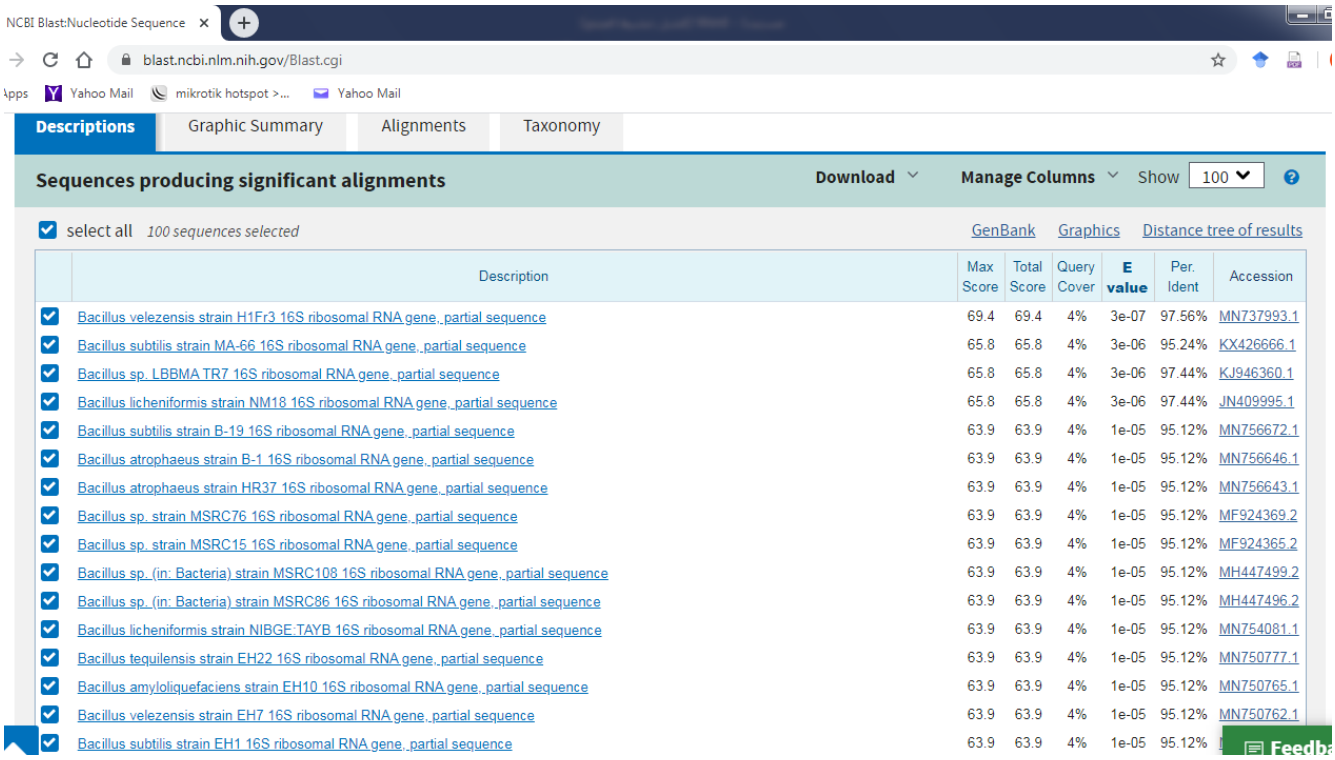

Figure (3-12) represented sequence results of *Bacillus velezensis* MN737993.1

A5AF

NTNNNGNGCGGNGCTATACATGCAGTCGAGCGGACAGATGGGAGCTTG
CTCCCTGATGGTAGCGTGGACGGGGGCTTCAAAGAGTAGATTTCCTTAG
GGAAATATATATTGCCCCTTTTCTTATACCCCCAGGGGGGAGCGACTCC
ACCTCCGTGCAAGTGGGAACATCTTATGCTCATTGCTCTTTTTTTTTTGG
GTTTGTGCTATGTCGGCTGCACTGGAGGTGTGGGGAGATTATCCCGACG
TCTTATCCGCCCCCCCCTTTTCAATGTGTATAACGAAGTTACCATCACTA
CTTCCCCCTCTCCCACATCCGGCATTGGCCCCGATACTGAACTTCGGTTA
TAAAAAACGCTCGTCTGCATCGCTCGGGGCCTACCGG
GAGAGTGACAATACGAGTACTTCATCAGAGGATAAATAGCAGTGATTTT
GTACCCGAGGTTTCGAACCCACAATCCCGCACCAAAGGCTCCCCGAGCT
GGCCCTACAAATTCCGCATCTTCTTCCGGCCATCTTCTATGTCGCCTTCA
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CCCCAAAAATGTGTTCCCACGTGTTGATTACCAGGGCCCCCAGATTCCTC

CTCCCTGAGGAGAGCCACTGTCGTAAGAACCTTAGTTAATCATATTCCG
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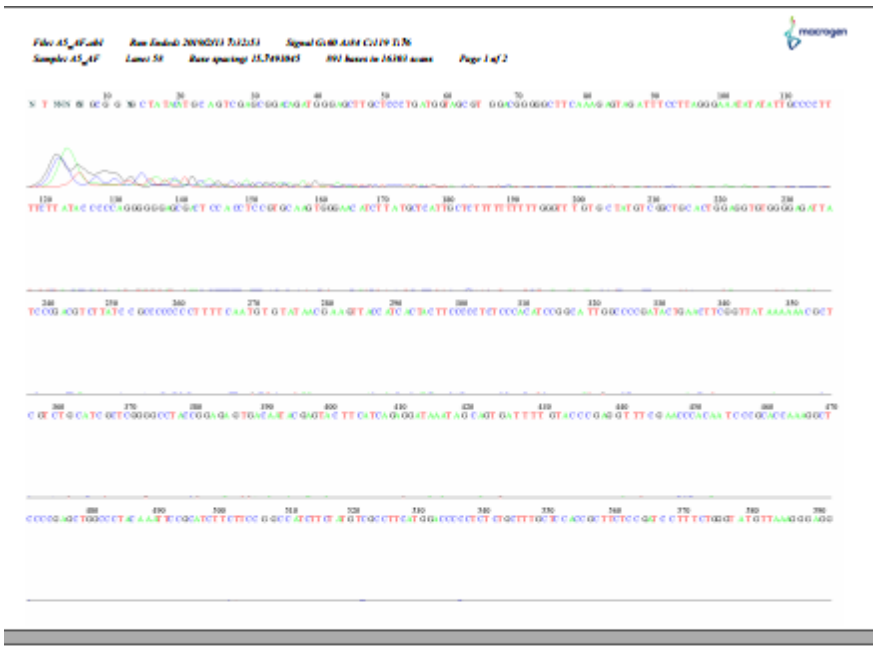


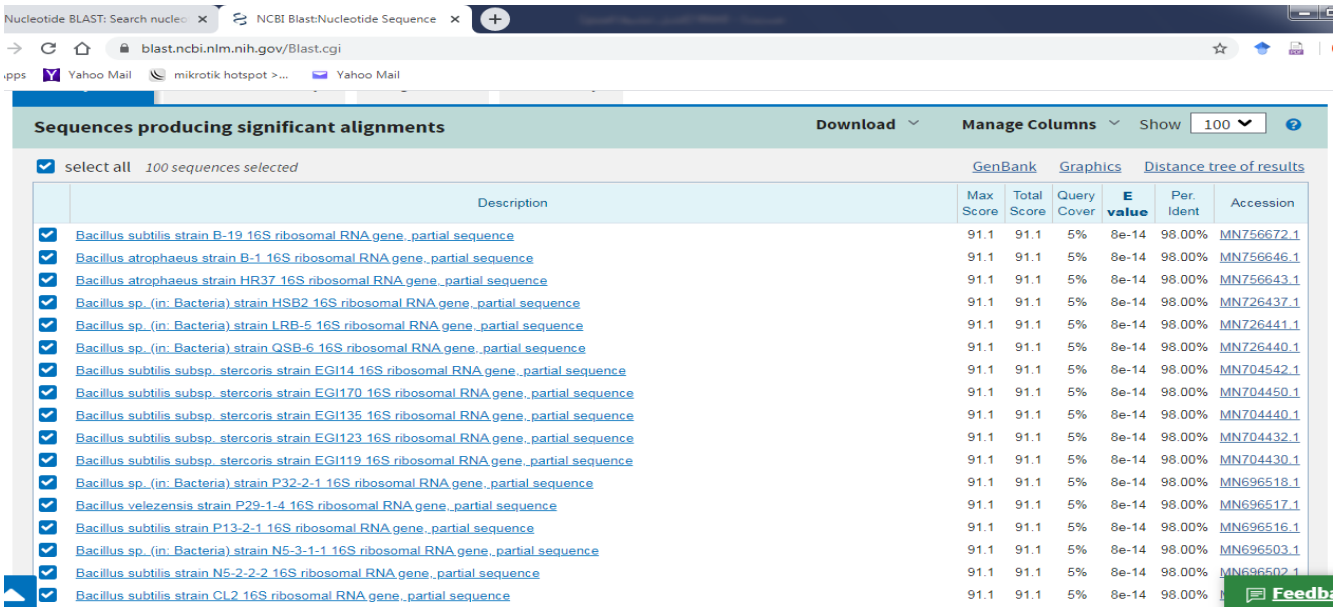


Figure (3-13) represented sequence results of *Bacillus subtilis* MN756672.

3-7 Growth parameters

Data on the growth performances, including weight gain (WG), feed conversion ratio (FCR), specific growth rate(SGR) and feed conversion efficiency(FCE) of common carp fed diets supplemented with or without *B. subtilis* are illustrated in Table 3.6 After the six weeks feeding period the inclusion of *B. subtilis* in the diet did not significantly affect the growth performance of common carp

Table(3-6) Growth performance of common carp fed the basal diet and the diet supplemented with the probiotic *Bacillus subtilis* for a period of 6 weeks

Measured parameter	Control	Probiotic
Initial mean weight (g)	77.4 ± 1.1^{a}	77.0 ± 0.6^{a}
Final mean weight (g)	97.9 ± 11.2^{a}	97.5 ± 5.1^{a}
Weight gain (g)	20.4 ± 11.5^{a}	20.5 ± 4.8^{a}
Feed conversion ratio	5.1 ± 2.7^{a}	6.4 ± 3.0^{a}
Specific growth rate g day $^{-1}$	0.58 ± 0.32^{a}	0.70 ± 0.13^{a}
Feed conversion efficiency	235.8 ± 38.4^{a}	230.8 ± 19.3^{a}

Means with the same letter in each row are not significantly different ($P > 0.05$). The data are expressed as the means ± S.D. (n = 7).

There were no significant differences between survival rate, final weight, weight gain, SGR, FCR or FCE of common carp fed the control and probiotic diets ($P > 0.05$). However, the specific growth rate (SGR), final weight and FCR showed an increasing trend in the probiotic treated common carp compared to the control

The current study failed to reveal any significant improvement in final gain in weight, FCR, SGR and PER in fish fed probiotic diet. These results were in agreement with previous studies that indicated probiotics and feed additives could be fed to fish without any adverse effect on growth indices. For example,(Ramos *et al.,* 2013) showed that growth performance in rainbow trout fed two commercial probiotics for 28 days revealed no significant improvement in growth performance rate. A study of (Cao, *et al.,* 2019) reported no significant differences in feed efficiency on rainbow trout fed *B. subtilis* supplemented diet for 70 days. In the same line, probiotics were given to juvenile dentex (*Dentex dentex*), significant influence for FCR was not reported (Hidalgo *et al*., 2006).

Several studies showed significant improvements in growth indices on common carp fed different genera of probiotic (Ahmadifar *et al.,* 2020; Ahmadifar *et al.,* 2019). The same tendency was reported with different fish species fed *Bacillus* spp. supplemented diet (Krishnan, 2014). Common carp were fed *B. subtilis* at two levels (1×10^3 -1×10^6) CFU ml^{-1} for 8 weeks and the findings indicated that weight gain, specific growth rate and protein efficiency were significantly higher in the group of fish fed probiotic diet at level 1×10^6 CFU ml^{-1} compared to the control group of fish (Koliaee *et al*., 2016)

In addition, improved production was reported using probiotic for shrimps with a significant lower conversion ratio to feed than in the control group (Castex *et al*., 2008). Also, a lower FCR was reported for Rohu fingerling Indian major carp *Labeo rohita* when they fed on probiotic diets (Mohapatra *et al*., 2013).

These observations are not in congruence with current data, due to such factors related to fish including age, type, physiological condition and genetic structure,

others related to probiotics including the probiotic feeding duration and dosage, the type and composition (i.e. mono-species or multi-species) of the probiotic, administration way of probiotics and type of supplementation plus the nature of relationship between probiotic and gut microbiota which could have affected the probiotics outcome in fish (Merrifield *et al.*, 2010; Nayak, 2010).

3-8 Interleukins

The levels of IL1 β in the serum of common carp fed treatment and control diets are presented in Figure (3. 14).The serum of probiotic-fed fish displayed significant increase of IL1β (P<0.004) compared to the control treatment

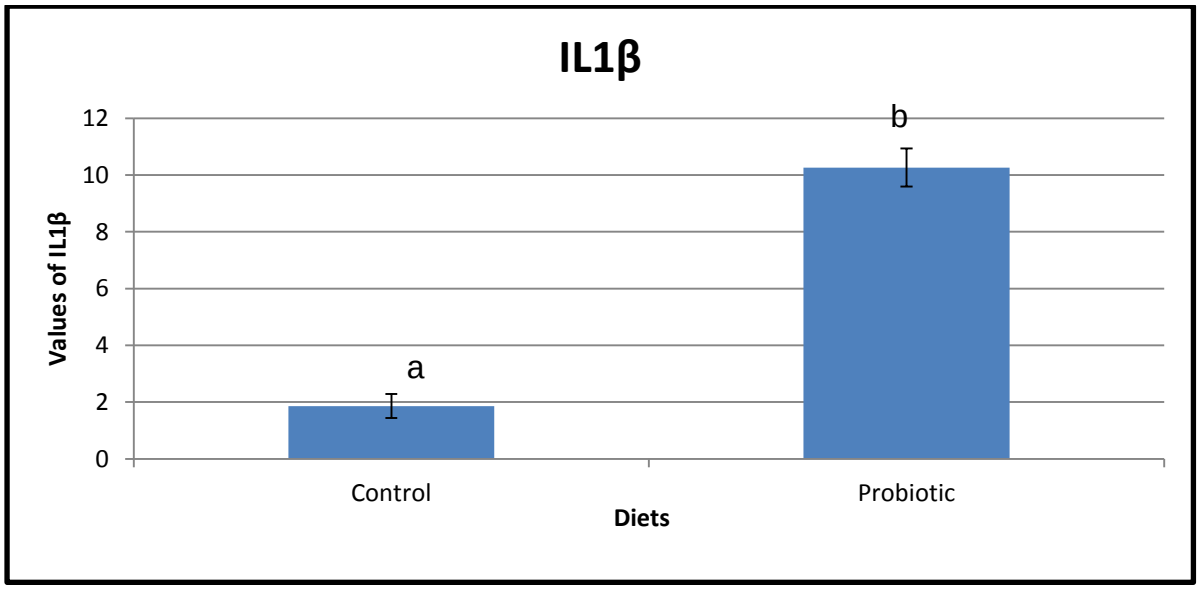


Figure (3. 14) Serum level of Interleukin 1 Beta(IL1β) in common carp fed treatment and control diets

Bars with the different letters between treatments are significantly different (P < 0.05). Values are mean ± SD represented by vertical bars of 7 replicates.

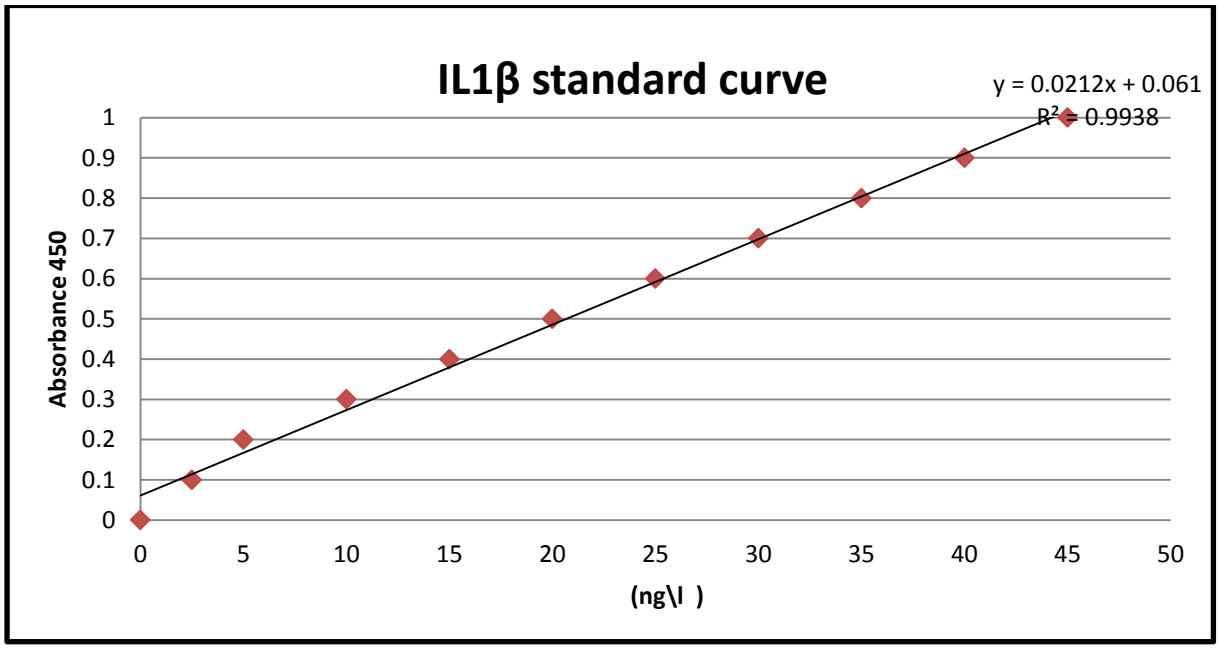
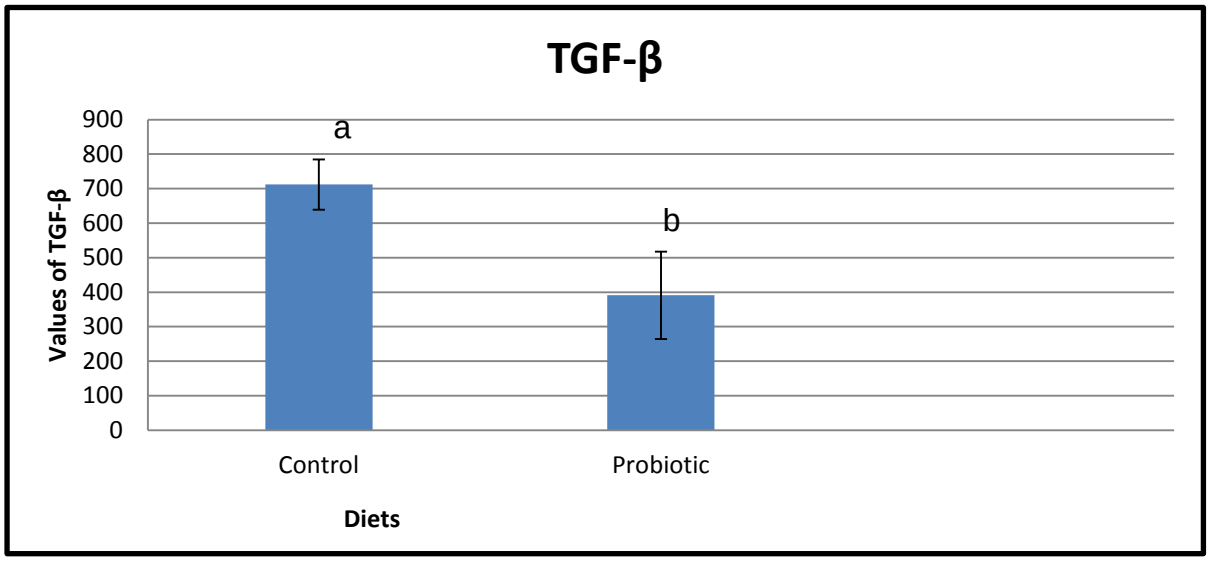


Figure (3. 15)Standard curve for detection of the level of IL-1 β

The levels of TGFβ in the serum of common carp fed treatment and control diets are presented in Figure (3.16) .In contrast,to IL1β result the anti-inflammatory cytokine genes, TGF-β, level was decreased in the serum of common carp the probiotic administration but no significant differences were observed (P = 0.05).



Figure(3. 16) Serum level Transforming growth factor beta(TGF-β)of common carp fed treatment and control diets

Bars with the different letters between treatments are significantly different ($P < 0.05$). Values are mean ± SD represented by vertical bars of 7 replicates.

It is commonly stated that pro-inflammatory cytokine IL-1*β* is primarily created from monocytes and macrophages and is considered a biomarker for testing immune regulation, by activating lymphocytes or by inducing the release of other cytokines which are able to elicit the activation of macrophages, NK cells and lymphocytes (Giri *et al*., 2015). In the current study, significant elevation in the levels of IL-1β was observed in *B. subtilis* fed fish compared to the control group of fish. Supporting this observation, previous studies revealed up-regulation of the expression of IL-1β after administration of probiotic feed additives in fish (Al-Hisnawi *et al*., 2019; Standen *et al*., 2016; Gioacchini *et al*., 2014). In contrast to the present study, in *Labeo rohita*, which were injected intraperitoneally with 0.1 mL of phosphate-buffered saline (PBS) containing the water-soluble fraction of purified biosurfactant at 200 (S200), or 300 (S300) µg mL^{-1} significantly down-regulated the levels of IL-1β (Giri *et al*., 2015).

Futhermore, rainbow trout fed with three freeze-dried probionts (*Lactobacillus rhamnosus*, *Enterococcus faecium* and *B. subtilis*) showed enhanced superoxide anion production, serum alternative complement activity and increased IL-1β, TNF and TGF-β expression in the spleen and the head of kidney (Panigrahi *et al.*, 2007). These results again suggest an increase in innate immunity and possibly regulatory mechanisms for mucosal tolerance (Merrifield *et al*., 2010 c ,d)

TGF-β is a pleiotropic cytokine that acts in different roles in the immune system which inhibits proliferation and differentiation of B and T cells and helps to down-regulate the expression of pro-inflammatory cytokines including IL-1β, TNF-α, and IFN-γ (He *et al*., 2011, Li and Flavell, 2008). Data obtained from the current study revealed that the levels of TGF-β in serum of common carp fed probiotics supplemented diet tend to be significantly decreased compared to the control group (P=0.05).

Consistent with these findings, previous studies demonstrated that feed supplemented with different species of probiotics bacteria (Al-Hisnawi *et al.*, 2019) and prebiotic (Hoseinifar *et al*., 2019; Wang *et al*., 2015) significantly down-regulated the expression of anti-inflammatory cytokine. In contrast to the present results, several studies revealed up-regulation of TGF-β in different fish species after probiotics bacteria supplementation (Standen *et al*., 2016; He *et al*., 2011; Giri *et al*., 2016).

The significant improvement of IL-1β in the present study suggests that probiotics supplemented diet could induce inflammatory response supported by elevation of WBC and inflammatory response which was not excessive or detrimental concomitantly with decreasing the levels of TGF-β in the *B. subtilis* fed fish compared to the control group. However, this hypothesis needs to be confirmed by histological observations of intestine tissue using light microscope.

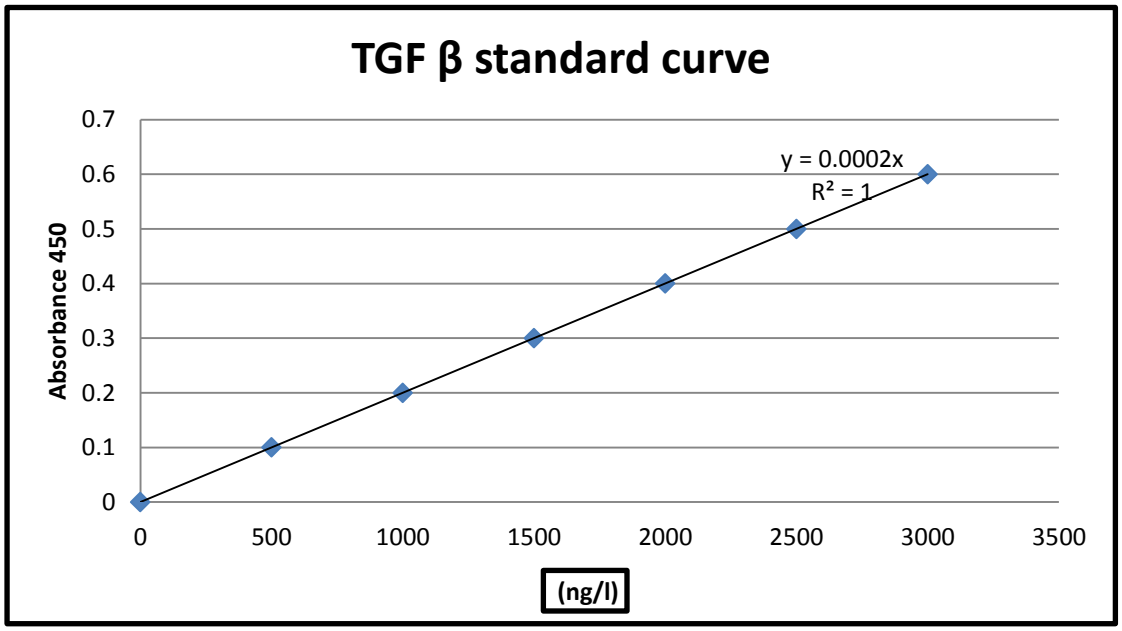


Figure (3. 17) Standard curve for detection of the level of TGF β

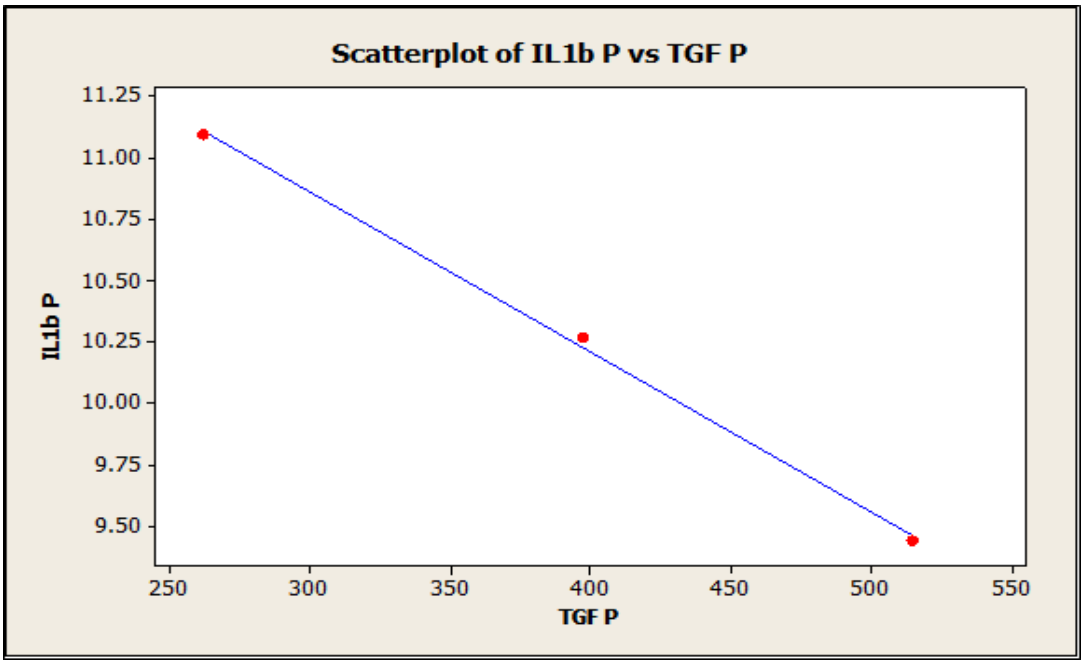


Figure (3-18) Negative correlation between IL 1 β and TGF β in probiotic (*P* =0.027)

Table (3-7) correlation coefficient between IL1 β and TGF β in control and probiotic groups

Table (3-7) showed that there is no correlation between different treatments, however negative correlation between IL1β and TGFβ was reported.

	IL1β control		IL1β probiotic	TGF β control
IL1β Probiotic	Type of correlation	-0.670	-	-
	P value	0.533	-	-
TGF β control	Type of correlation	0.308	0.500	-
	P value	0.800	0.667	-
TGF β probiotic	Type of correlation	0.638	-0.999	-0.536
	P value	0.559	0.027	0.640

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Conclusions and Recominandations

Conclusions

1. The addition of *B. subtilis* to commercial diet had a potential role in modulating of the intestinal microbiota of common carp *B. subtilis* accomplish a competitive role against harmful bacteria.
2. intestinal bacteria of common carp are mainly composed of Proteobacteria followed by Firmicutes
3. The probiotic application enhanced the immune response in terms of increase of pro-inflammatory cytokines and decrease of anti-inflammatory cytokines.
4. No detrimental effects of growth performance were observed.

Recominandations

1-Culture-independent techniques including PCR-DGGE and PCR-clone libraries should be used in future studies in order to give clear picture for microbial community in common carp in Iraq.

2-Design a probe labelled with fluorescent dye to identify *B. subitils* under fluorescence microscope.

3- Quantitative PCR would be used to determine the effects of probiotic modulation on the host localised immune response by the investigation of a wide range of pro- and anti- inflammatory cytokines as well as biomarkers of stress (HSP70), apoptosis (casp-3) and programmed cell death (PCNA). The immune status should be further evaluated by the investigation of lysozyme enzyme, complement system and respiratory burst activity.

4-Disease challenge studies would be useful to investigate the beneficial effects of *B. subtilis* in common carp.

5-Electron and light microscopes should be used in the future works for investigation of histological changes of the GIT after diets feeding.

6- However, further studies need to be conducted to determine the immunostimulant effects, mechanisms of action and the most appropriate levels of *B. subtilis* for the best growth and immune conditions.

7-Legislation of some lows are needed to obligate owners of local fish farming to use aprobiotic supplemented diet instead of using antibiotics.

8-Ministry of aqriculture has to get benefits from this type of studies and producing diets supplementation with probiotic bacteria as a amin feeding regime of meet-producing animals,including fish ,to enhance the immune system of these animals ,to the extent that prevents the use of antibiotics in treating diseaese .

References

Abdel - Tawwab, M., & Ahmad, M. H. (2009). Live Spirulina (Arthrospira platensis) as a growth and immunity promoter for Nile tilapia, Oreochromis niloticus (L.), challenged with pathogenic Aeromonas hydrophila. *Aquaculture Research*, *40*(9), 1037-1046.

Agouz, H. M., & Anwer, W. (2011). Effect of Biogen® and Myco-Ad® on the growth performance of common carp (Cyprinus carpio) fed a mycotoxin contaminated aquafeed. *Journal of Fisheries and Aquatic Science*, *6*(3), 334.

Aguirre-Guzmán, G., Lara-Flores, M., Sánchez-Martínez, J. G., Campa-Córdova, A. I., & Luna-González, A. (2012). The use of probiotics in aquatic organisms: A review. *African Journal of Microbiology Research*, *23*, 4845-4857.

Ahmadifar, E., Moghadam, M. S., Dawood, M. A., & Hoseinifar, S. H. (2019). Lactobacillus fermentum and/or ferulic acid improved the immune responses, antioxidative defence and resistance against Aeromonas hydrophila in common carp (Cyprinus carpio) fingerlings. *Fish & shellfish immunology*, *94*, 916-923.

Ahmadifar, E., Sadegh, T. H., Dawood, M. A., Dadar, M., & Sheikhzadeh, N. (2020). The effects of dietary Pediococcus pentosaceus on growth performance, hemato-immunological parameters and digestive enzyme activities of common carp (Cyprinus carpio). *Aquaculture*, *516*, 734656.

Akter, M. N., Parvez, I., & Patwary, Z. P. (2016). Beneficial effects of probiotics in aquaculture. *International Journal of Fisheries and Aquatic Studies*, *4*(5), 494-499.

Alakomi, H. L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., & Helander, I. M. (2000). Lactic acid permeabilizes gram-

negative bacteria by disrupting the outer membrane. *Applied and environmental microbiology*, *66*(5), 2001-2005 .

Al-Dohail, M. A., Hashim, R., & Aliyu-Paiko, M. (2009). Effects of the probiotic, *Lactobacillus acidophilus*, on the growth performance, haematology parameters and immunoglobulin concentration in African Catfish (Clarias gariepinus, Burchell 1822) fingerling. *Aquaculture Research*, *40*(14), 1642-1652.

Al-Harbi, A. H., & Uddin, M. N. (2004). Seasonal variation in the intestinal bacterial flora of hybrid tilapia (*Oreochromis niloticus*× *Oreochromis aureus*) cultured in earthen ponds in Saudi Arabia. *Aquaculture*, *229*(1-4), 37-44.

Al-Hisnawi, A., Ringø, E., Davies, S. J., Waines, P., Bradley, G., & Merrifield, D. L. (2015). First report on the autochthonous gut microbiota of brown trout (S almo trutta Linnaeus). *Aquaculture Research*, *46*(12), 2962-2971.

Al-Hisnawi, A., Rodiles, A., Rawling, M. D., Castex, M., Waines, P., Gioacchini, G., ... & Merrifield, D. L. (2019). Dietary probiotic Pediococcus acidilactici MA18/5M modulates the intestinal microbiota and stimulates intestinal immunity in rainbow trout (Oncorhynchus mykiss). *Journal of the World Aquaculture Society*, *50*(6), 1133-1151.

Allameh, S. K., Ringø, E., Yusoff, F. M., Daud, H. M., & Ideris, A. (2014). Properties of Enterococcus faecalis, a new probiotic bacterium isolated from the intestine of snakehead fish (Channa striatus Bloch). *African Journal of Microbiology Research*, *8*(22), 2215-2222.

Al-Mahmood, S. S., Bakir, D. W., & Hussen, S. H. (2017). Gross and histopathological study on common carp Cyprinus carpio L. diseases in

rearing culturing ponds in Kirkuk Province–Iraq. *The Iraqi Journal of Veterinary Medicine*, *41*(1), 109-117..

Alou, M. T., Rathored, J., Khelaifia, S., Michelle, C., Brah, S., Diallo, B. A., ... & Lagier, J. C. (2015). Bacillus rubiinfantis sp. nov. strain mt2T, a new bacterial species isolated from human gut. *New microbes and new infections*, *8*, 51-60.

Aly, S. M., Ahmed, Y. A. G., Ghareeb, A. A. A., & Mohamed, M. F. (2008). Studies on Bacillus subtilis and Lactobacillus acidophilus, as potential probiotics, on the immune response and resistance of Tilapia nilotica (Oreochromis niloticus) to challenge infections. *Fish & shellfish immunology*, *25*(1-2), 128-136.

Amuguni, H., & Tzipori, S. (2012). Bacillus subtilis: a temperature resistant and needle free delivery system of immunogens. *Human vaccines & immunotherapeutics*, *8*(7), 979-986..

Anton-Pardo, M., Hlaváč, D., Másílko, J., Hartman, P., & Adámek, Z. (2014). Natural diet of mirror and scaly carp (Cyprinus carpio) phenotypes in earth ponds. *Journal of Vertebrate Biology*, *63*(4), 229-237.

Arellano-Carbajal, F., & Olmos-Soto, J. (2002). Thermostable α-1, 4-and α-1, 6-glucosidase enzymes from Bacillus sp. isolated from a marine environment. *World Journal of Microbiology and Biotechnology*, *18*(8), 791-795**.**

Arthur, J. R., & Subasinghe, R. P. (2002). Potential adverse socio-economic and biological impacts of aquatic animal pathogens due to hatchery-based enhancement of inland open-water systems, and possibilities for their minimisation. *FAO Fisheries Technical Paper*, *406*, 113-126.

Austin, B., Stuckey, L. F., Robertson, P. A. W., Effendi, I., & Griffith, D R. W. (1995). A probiotic strain of Vibrio alginolyticus effective in reducing diseases caused by Aeromonas salmonicida, Vibrio anguillarum and Vibrio ordalii. *Journal of Fish Diseases*, *18*(1), 93-96.

Bagheri, T., Hedayati, S. A., Yavari, V., Alizade, M., & Farzanfar, A. (2008). Growth, survival and gut microbial load of rainbow trout (Onchorhynchus mykiss) fry given diet supplemented with probiotic during the two months of first feeding. *Turkish Journal of Fisheries and Aquatic Sciences*, *8*(1), 43-48.

Bairagi, A., Ghosh, K. S., Sen, S. K., & Ray, A. K. (2002). Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquaculture International*, *10*(2), 109-121.

Bajer, P. G., & Sorensen, P. W. (2015). Effects of common carp on phosphorus concentrations, water clarity, and vegetation density: a whole system experiment in a thermally stratified lake. *Hydrobiologia*, *746*(1), 303-311.

Balcazar, J. L. (2003). Evaluation of probiotic bacterial strains in Litopenaeus vannamei. Final Report. *National Center for Marine and Aquaculture Research, Guayaquil, Ecuador*.

Balcázar, J. L., De Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., & Muzquiz, J. L. (2006). The role of probiotics in aquaculture. *Veterinary microbiology*, *114*(3-4), 173-186.

Balcázar, J. L., De Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Calvo, A. C., Márquez, I., ... & Muzquiz, J. L. (2007). Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (Salmo trutta). *British journal of nutrition*, *97*(3), 522-527.

Bandyopadhyay, P., & Mohapatra, P. K. D. (2009). Effect of a probiotic bacterium Bacillus circulans PB7 in the formulated diets: on growth, nutritional quality and immunity of Catla catla (Ham.). *Fish physiology and biochemistry*, *35*(3), 467-478.

Banerjee, S., Khatoon, H., Shariff, M., & Yusoff, F. M. (2010). Enhancement of Penaeus monodon shrimp postlarvae growth and survival without water exchange using marine Bacillus pumilus and periphytic microalgae. *Fisheries Science*, *76*(3), 481-487.

Barbosa, T. M., Serra, C. R., La Ragione, R. M., Woodward, M. J., & Henriques, A. O. (2005). Screening for Bacillus isolates in the broiler gastrointestinal tract. *Appl. Environ. Microbiol.*, *71*(2), 968-978.

Barus V, Peaz M, Kohlmann K. (2001). *Cyprinus carpio* (Linnaeus, 1758). In Banarescu PM, Paepke HJ, editor. The freshwater fishes of Europe, v. 5/III; Cyprinidae 2/III, and Gasterosteidae. Germany: AULA-G GmbH Wiebelsheim; p. 85–179

Bassey, S. E. (2011). A Concise Dictionary of Parasitology. *Zetus Concepts*, *978-2954-40*, *3*(1), 115.

Bates, J. M., Mittge, E., Kuhlman, J., Baden, K. N., Cheesman, S. E., & Guillemin, K. (2006). Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. *Developmental biology*, *297*(2), 374-386.

Berdy, J. (2005). Bioactive microbial metabolites. *The Journal of antibiotics*, *58*(1), 1-26.

Bierbaum, G., & Sahl, H. G. (2009). Lantibiotics: mode of action, biosynthesis and bioengineering. *Current pharmaceutical biotechnology*, *10*(1), 2-18.

Brown, M. (2011). Modes of action of probiotics: recent developments. *Journal of animal and veterinary advances*, *10*(14), 1895-1900.

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental microbiology*, *8*(7), 1137-1144.

Cai, Z., Gao, C., Li, L., & Xing, K. (2010). Bipolar properties of red seabream (Pagrus major) transforming growth factor-β in induction of the leucocytes migration. *Fish & shellfish immunology*, *28*(4), 695-700.

Capriles, V. D., Silva, K. E. A. D., & Fisberg, M. (2005). Prebióticos, probióticos e simbióticos: nova tendência no mercado de alimentos funcionais. *Nutrição Brasil*, *4*(6), 327-335.

Cao, H., Yu, R., Zhang, Y., Hu, B., Jian, S., Wen, C., ... & Yang, G. (2019). Effects of dietary supplementation with β-glucan and Bacillus subtilis on growth, fillet quality, immune capacity, and antioxidant status of Pengze crucian carp (Carassius auratus var. Pengze). *Aquaculture*, *508*, 106-112.

Carda-Diéguez, M., Mira, A., & Fouz, B. (2014). Pyrosequencing survey of intestinal microbiota diversity in cultured sea bass (Dicentrarchus labrax) fed functional diets. *FEMS microbiology ecology*, *87*(2), 451-459.

Castex, M., Chim, L., Pham, D., Lemaire, P., Wabete, N., Nicolas, J. L., ... & Mariojouls, C. (2008). Probiotic P. acidilactici application in shrimp Litopenaeus stylirostris culture subject to vibriosis in New Caledonia. *Aquaculture*, *275*(1-4), 182-193.

Cherif, A., Ouzari, H., Daffonchio, D., Cherif, H., Ben Slama, K., Hassen, A., ... & Boudabous, A. (2001). Thuricin 7: a novel bacteriocin produced by Bacillus thuringiensis BMG1. 7, a new strain isolated from soil. *Letters in Applied Microbiology*, *32*(4), 243-247.

Collado, M. C., Gueimonde, M., Hernandez, M., Sanz, Y., & Salminen, S. (2005). Adhesion of selected Bifidobacterium strains to human intestinal

mucus and the role of adhesion in enteropathogen exclusion. *Journal of food protection*, *68*(12), 2672-2678 .

Cutting, S. M. (2011). Bacillus probiotics. *Food, microbiology*, *28*(2), 214-220.

Das, A., Acharya, S., Behera, B. K., Paria, P., Bhowmick, S., Parida, P. K., & Das, B. K. (2018). Isolation, identification and characterization of Klebsiella pneumoniae from infected farmed Indian Major Carp Labeo rohita (Hamilton 1822) in West Bengal, India. *Aquaculture*, *482*, 111-116.

Das, A., Nakhro, K., Chowdhury, S. and Kamilya, D. (2013). Effectsof potential probiotic Bacillus amyloliquifaciens FPTB16 on systemicand cutaneous mucosal immune responses and disease resistance ofcatla (Catla catla), *Fish Shellfish Immunol.*, 35: 1547-1553.

Dawood, M. A., & Koshio, S. (2016). Recent advances in the role of probiotics and prebiotics in carp aquaculture: a review. *Aquaculture*, *454*, 243-251.

Dawood, M. A., Koshio, S., & Esteban, M. Á. (2018). Beneficial roles of feed additives as immunostimulants in aquaculture: a review. *Reviews in Aquaculture*, *10*(4), 950-974.

Dawood, M. A., Koshio, S., Ishikawa, M., Yokoyama, S., El Basuini, M. F., Hossain, M. S., ... & Moss, A. S. (2016). Effects of dietary supplementation of Lactobacillus rhamnosus or/and Lactococcus lactis on the growth, gut microbiota and immune responses of red sea bream, Pagrus major. *Fish & Shellfish Immunology*, *49*, 275-285.

Deeseenthum, S., Leelavatcharamas, V., & Brooks, J. D. (2007). Effect of Feeding Bacillus sp. As Probiotic Bacteria on Growth of Giant Freshwater Prawn(Macrobrachium rosenbergii de Man). *Pakistan Journal of Biological Sciences*, *10*(9), 1481-1485.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., and Bossier, P. (2007). Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. *Trends Biotechnol.* 25, 472–479.

Dehler, C. E., Secombes, C. J., & Martin, S. A. (2017). Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (Salmo salar L). *Aquaculture*, *467*, 149-157.

Dicks, L., & Botes, M. (2010). Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. *Beneficial microbes*, *1*(1), 11-29.

Dutilh, B. E., Neveling, K., Kwint, M. P., Veltman, J. A., Flik, G., Jetten, M. S., & den Camp Op, H. J. (2011). Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (Cyprinus carpio L.). *AMB Express*, *1*(1), 1-9

El-Haroun, E. R., Goda, A. S., & Kabir Chowdhury, M. A. (2006). Effect of dietary probiotic Biogen® supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia Oreochromis niloticus (L.). *Aquaculture Research*, *37*(14), 1473-1480.

Elshaghabee, F. M., Rokana, N., Gulhane, R. D., Sharma, C., & Panwar, H. (2017). Bacillus as potential probiotics: status, concerns, and future perspectives. *Frontiers in microbiology*, *8*, 1490.

Elward, K., & Gasque, P. (2003). "Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system. *Molecular immunology*, *40*(2-4), 85-94.

Essa, M. A., El-Serafy, S. S., El-Ezabi, M. M., Daboor, S. M., Esmael, N. A., & Lall, S. P. (2010). Effect of different dietary probiotics on growth, feed utilization and digestive enzymes activities of Nile tilapia,

Oreochromis niloticus. *Journal of the Arabian Aquaculture Society*, *5*(2), 143-162.

Faramarzi, M., Kiaalvandi, S., Lashkarbolooki, M., & Iranshahi, F. (2011). The investigation of Lactobacillus acidophilus as probiotics on growth performance and disease resistance of rainbow trout (Oncorhychus mykiss). *American-Eurasian journal of scientific Research*, *6*(1), 32-38.

Farzanfar, A. (2006). The use of probiotics in shrimp aquaculture. *FEMS Immunology & Medical Microbiology*, *48*(2), 149-158.

Farzanfar, A., Lashtooaghaee, G., Alizadeh, M., Bayati, M., & Ghorbani, R. (2009, May). Effect of using Bacillus subtilis and Bacillus licheniformis as probiotic on growth parameters, survival and carcass quality in rainbow trout, Oncorhynchus mykiss (Walbaum) fry. In *The first national coldwater fishes conference, Tonekabon-Iran,(12-14 May 2009)* (Vol. 154).

Firdaus-Nawi, M., & Zamri-Saad, M. (2016). Major Components of Fish Immunity: A Review. *Pertanika Journal of Tropical Agricultural Science*, *39*(4).

Food and Agriculture organization(FAO) (2013). Fishstate plus: Universal software for fishery statistical time series (available at: www.fao.org/fi/statist/fisoft/fishplus.asp).

Francis-Floyd, R., & Wellborn, T. L. (2005). *Introduction to fish health management*. Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florid.

Fuller, R. (1989). A review: probiotics in man and animals. *Journal of Applied Bacteriology*, *66*(5), 365-378.

Gatesoupe, F. J. (2008). Updating the importance of lactic acid bacteria in fish farming: natural occurrence and probiotic treatments. *Journal of molecular microbiology and biotechnology*, *14*(1-3), 107-114..

Gioacchini, G., Giorgini, E., Olivotto, I., Maradonna, F., Merrifield, D. L., & Carnevali, O. (2014). The influence of probiotics on zebrafish Danio rerio innate immunity and hepatic stress. *Zebrafish*, *11*(2), 98-106.

Giri, S. S., Sen, S. S., Chi, C., Kim, H. J., Yun, S., Park, S. C., & Sukumaran, V. (2015). Effect of cellular products of potential probiotic bacteria on the immune response of Labeo rohita and susceptibility to Aeromonas hydrophila infection. *Fish& shellfish immunology*, *46*(2), 716-722.

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., ... & Toulmin, C. (2010). Food security: the challenge of feeding 9 billion people. *science*, *327*(5967), 812-818.

González-Rodríguez, I., Sánchez, B., Ruiz, L., Turroni, F., Ventura, M., Ruas-Madiedo, P., ... & Margolles, A. (2012). Role of extracellular transaldolase from Bifidobacterium bifidum in mucin adhesion and aggregation. *Appl. Environ. Microbiol.*, *78*(11), 3992-3998.

Grisez, L., & Ollevier, F. (1995). Vibrio (Listonella) anguillarum infection in marine fish larviculture. *Larvi*, *91*, 497.

Grześkowiak, Ł., Collado, M. C., Vesterlund, S., Mazurkiewicz, J., & Salminen, S. (2011). Adhesion abilities of commensal fish bacteria by use of mucus model system: Quantitative analysis. *Aquaculture*, *318*(1-2), 33-36.

Günther, J., & Jiménez-Montealegre, R. (2004). Efecto del probiótico Bacillus subtilis sobre el crecimiento y alimentación de tilapia (Oreochromis niloticus) y langostino (Macrobrachium rosenbergii) en laboratorio. *Revista de biología tropical*, *52*(4), 937-943.

Guo, M., Yang, W., Wu, F., Hao, G., Li, R., Wang, X., ... & Chai, T. (2017). Colonization, mortality, and host cytokines response to enterohemorrhagic Escherichia coli in rabbits. *Oncotarget*, *8*(55), 93426.

Gutowska, M. A., Drazen, J. C., & Robison, B. H. (2004). Digestive chitinolytic activity in marine fishes of Monterey Bay, California. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *139*(3), 351-358.

Haddad, G., Hanington, P. C., Wilson, E. C., Grayfer, L., & Belosevic, M. (2008). Molecular and functional characterization of goldfish (Carassius auratus L.) transforming growth factor beta. *Developmental & comparative immunology*, *32*(6), 654-663.

Hagi, T., Tanaka, D., Iwamura, Y., & Hoshino, T. (2004). Diversity and seasonal changes in lactic acid bacteria in the intestinal tract of cultured freshwater fish. *Aquaculture*, *234*(1-4), 335-346.

Hai, N. V. (2015). The use of probiotics in aquaculture. *Journal of applied microbiology*, *119*(4), 917-935.

Hemaiswarya, S., Raja, R., Ravikumar, R., & Carvalho, I. S. (2013). Mechanism of action of probiotics. *Brazilian archives of Biology and technology*, *56*(1), 113-119.

Heo, W. S., Kim, Y. R., Kim, E. Y., Bai, S. C., & Kong, I. S. (2013). Effects of dietary probiotic, Lactococcus lactis subsp. lactis I2, supplementation on the growth and immune response of olive flounder (Paralichthys olivaceus). *Aquaculture*, *376*, 20-24.

He S, Liu W, Zhou Z, Mao W, Ren P, et al. (2011). Evaluation of probiotic strain Bacillus subtilis C-3102 as a feed supplement for koi carp (Cyprinus carpio) J Aquac Res Development S1:005. doi:10.4172/2155

Hidalgo, M. C., Skalli, A., Abellán, E., Arizcun, M., & Cardenete, G. (2006). Dietary intake of probiotics and maslinic acid in juvenile dentex

(Dentex dentex L.): effects on growth performance, survival and liver proteolytic activities. *Aquaculture nutrition*, *12*(4), 256-266.

Hoseinifar, S. H., Zou, H. K., Paknejad, H., Hajimoradloo, A., & Van Doan, H. (2019). Effects of dietary white-button mushroom powder on mucosal immunity, antioxidant defence, and growth of common carp (Cyprinus carpio). *Aquaculture*, *501*, 448-454.

Huang, Q., Xu, X., Mao, Y. L., Huang, Y., Rajput, I. R., & Li, W. F. (2013). Effects of B acillus subtilis B 10 spores on viability and biological functions of murine macrophages. *Animal Science Journal*, *84*(3), 247-252.

Huising, M. O., Stet, R. J., Savelkoul, H. F., & Verburg-van Kemenade, B. L. (2004). The molecular evolution of the interleukin-1 family of cytokines; IL-18 in teleost fish. *Developmental & Comparative Immunology*, *28*(5), 395-413

Human, T. (2012). Project M. Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207-14.

Idowu, T. A., & Sogbesan, O. A. (2017). Synthetic chemical application in aquaculture production. *International Journal Environmental & Agricultural Science*, *1*(2), 1-10.

Irianto, A., & Austin, B. (2002a). Use of probiotics to control furunculosis in rainbow trout, Oncorhynchus mykiss (Walbaum). *Journal of fish diseases*, *25*(6), 333-342.

Irianto, A., & Austin, B. (2002b). Probiotics in aquaculture. *Journal of fish diseases*, *25*(11), 633-642.

Jacobson-Kram, D., & Keller, K. A. (2001). Toxicology Testing Handbook: Principles. *Applications and Data interpretation. 2nd Edn., Marcel Decker, New York.*

Kadowaki, T., Yasui, Y., Takahashi, Y., Kohchi, C., Soma, G. I., & Inagawa, H. (2009). Comparative immunological analysis of innate immunity activation after oral administration of wheat fermented extract to teleost fish. *Anticancer research*, *29*(11), 4871-4877.

Kaktcham, P. M., Temgoua, J. B., Zambou, F. N., Diaz-Ruiz, G., Wacher, C., & de Lourdes Pérez-Chabela, M. (2017). Quantitative analyses of the bacterial microbiota of rearing environment, tilapia and common carp cultured in earthen ponds and inhibitory activity of its lactic acid bacteria on fish spoilage and pathogenic bacteria. *World Journal of Microbiology and Biotechnology*, *33*(2), 32.

Kamei, Y., Yoshimizu, M., Ezura, Y., & Kimura, T. (1988). Screening of bacteria with antiviral activity from fresh water salmonid hatcheries. *Microbiology and immunology*, *32*(1), 67-73.

Kamgar, M., & Ghane, M. (2014). Studies on Bacillus subtilis, as potential probiotics, on the hematological and biochemical parameters of rainbow trout, Oncorhynchus mykiss (Walbaum). *Journal of Applied and Environmental Microbiology*, *2*(5), 203-207.

Kloskowski J (2011) Impact of common carp *Cyprinus carpio* on aquatic communities: direct trophic effects versus habitat deterioration. *Fundamental and Applied Limnology-Archiv furHydrobiologie*, *178*(3), 245

Koliaee, Z., Aberoumand, A., & Ziaei-Nejad, S. (2016). The effect of probiotic Bacillus subtilis bacteria extracted from the intestine of common carp (Cyprinus carpio) on growth performance and survival of the fish. *Journal of Aquaculture Development*, *10*(2), 108-99.

Kolndadacha, O. D., Adikwu, I. A., Okaeme, A. N., Atiribom, R. Y., Mohammed, A., & Musa, Y. M. (2011). The role of probiotics in

aquaculture in Nigeria-a review. *Continental Journal of Fisheries and Aquatic Science*, *5*(1), 8-15 .

Korkea-Aho, T. L., Heikkinen, J., Thompson, K. D., Von Wright, A., & Austin, B. (2011). Pseudomonas sp. M174 inhibits the fish pathogen Flavobacterium psychrophilum. *Journal of applied microbiology*, *111*(2),266-277.

Kotb, E. (2015). Purification and partial characterization of serine fibrinolytic enzyme from Bacillus megaterium KSK-07 isolated from kishk, a traditional Egyptian fermented food. *Applied biochemistry and microbiology*, *51*(1), 34-43.

Krishnan, R. (2014). Probiotic potential of Bacillus species isolated from freshwater fish Anabas testudineus in Labeo rohita. *International Journal Research Development*, *1*, 46-50.

Kumar, R., Mukherjee, S. C., Prasad, K. P., & Pal, A. K. (2006). Evaluation of Bacillus subtilis as a probiotic to Indian major carp Labeo rohita (Ham.). *Aquaculture Research*, *37*(12), 1215-1221

Kumar, R., Mukherjee, S. C., Ranjan, R., & Nayak, S. K. (2008). Enhanced innate immune parameters in Labeo rohita (Ham.) following oral administration of Bacillus subtilis. *Fish & shellfish immunology*, *24*(2), 168-172.

Lakshmi, B., Viswanath, B., & Sai Gopal, D. V. R. (2013). Probiotics as antiviral agents in shrimp aquaculture. *Journal of pathogens*, 2013.

Lakshmanan, R., & Soundarapandian, P. (2008). Effect of commercial probiotics on large scale culture of black tiger shrimp Penaeus monodon (Fabricius). *Research journal of microbiology*, *3*(3), 198-203.

Lara-Flores, M., & Aguirre-Guzman, G. (2009). The use of probiotic in fish and shrimp aquaculture. A review. *Probiotics: production, evaluation and uses in animal feed. Research Signpost, Kerala*, 75-89.

. **Li, B., Samanta, A., Song, X., Furuuchi, K., Iacono, K. T., Kennedy, S., ... & Greene, M. I. (2006).** FOXP3 ensembles in T-cell regulation. *Immunological reviews*, *212*(1), 99-113.

Liewellyn, M. S., Boutin, S., Hoseinifar, S. H., & Derome, N. (2014). Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in microbiology*, *5*, 207.

Li, J., Tan, B., & Mai, K. (2009). Dietary probiotic Bacillus OJ and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (Litopenaeus vannamei). *Aquaculture*, *291*(1-2), 35-40.

Li, M. O., & Flavell, R. A. (2008). TGF-β: a master of all T cell trades. *Cell*, *134*(3), 392-404.

Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K. L., & Flavell, R. A. (2006). Transforming growth factor-β regulation of immune responses. *Annu. Rev. Immunol.*, *24*, 99-146.

Li, X., Yan, Q., Ringø, E., Wu, X., He, Y., & Yang, D. (2016). The influence of weight and gender on intestinal bacterial community of wild largemouth bronze gudgeon (Coreius guichenoti, 1874). *BMC microbiology*, *16*(1), 1-8.

Low, C., Wadsworth, S., Burrells, C., & Secombes, C. J. (2003). Expression of immune genes in turbot (Scophthalmus maximus) fed a nucleotide-supplemented diet. *Aquaculture*, *221*(1-4), 23-40. .

Lu, D. Q., Bei, J. X., Feng, L. N., Zhang, Y., Liu, X. C., Wang, L., ... & Lin, H. R. (2008). Interleukin-1β gene in orange-spotted grouper, Epinephelus coioides: Molecular cloning, expression, biological activities and signal transduction. *Molecular immunology*, *45*(4), 857-867.

MacFaddin, J. F. (2000). Biochemical Tests for Identification of Bacteria of Medical Importance.

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish & shellfish immunology*, *20*(2), 137-151.

Makras, L., Triantafyllou, V., Fayol-Messaoudi, D., Adriany, T., Zoumpopoulou, G., Tsakalidou, E., ... & De Vuyst, L. (2006). Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards Salmonella enterica serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. *Research in Microbiology*, *157*(3), 241-247.

Manjappa, K., Keshavanath, P., & Gangadhara, B. (2011). Influence of sardine oil supplemented fish meal free diets on common carp (Cyprinus carpio) growth, carcass composition and digestive enzyme activity. *Journal of Fisheries and Aquatic Science*, *6*(6), 604-613.

Martínez Cruz, P. (2012). I bánez AL, Monroy Hermosillo OA, Ramírez Saad HC. *Use of probiotics in aquaculture. ISRN Microbiol*, *2012*, 1e13.

Meng, X. L., Li, S., Qin, C. B., Zhu, Z. X., Hu, W. P., Yang, L. P., & Nie, G. X. (2018). Intestinal microbiota and lipid metabolism responses in the common carp (Cyprinus carpio L.) following copper exposure. *Ecotoxicology and environmental safety*, *160*, 257-264.

Merrifield DL, Harper GM, Dimitroglou A, Ringo E, Davies SJ (2010a). Possible influence of probiotic adhesion to intestinal mucosa on the activity and morphology of rainbow trout (O*ncorhyncus mykiss*) enterocytes. . *Aquaculture Research*, *41*(8), 1268-1272.

Merrifield, D. L., Bradley, G., Baker, R. T. M., & Davies, S. J. (2010d). Probiotic applications for rainbow trout (Oncorhynchus mykiss Walbaum) II. Effects on growth performance, feed utilization, intestinal

microbiota and related health criteria post antibiotic treatment. *Aquaculture nutrition*, *16*(5), 496-503.

Merrifield, D. L., Dimitroglou, A., Bradley, G., Baker, R. T. M., & Davies, S. J. (2010c). Probiotic applications for rainbow trout (Oncorhynchus mykiss Walbaum) I. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria. *Aquaculture Nutrition*, *16*(5), 504-510

Merrifield, D. L., Dimitroglou, A., Foey, A., Davies, S. J., Baker, R. T., Bøgwald, J., ... & Ringø, E. (2010b). The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture*, *302*(1-2), 1-18.

Mo, W. Y., Chen, Z., Leung, H. M., & Leung, A. O. W. (2017). Application of veterinary antibiotics in China's aquaculture industry and their potential human health risks. *Environmental Science and Pollution Research*, *24*(10), 8978-8989.

Mohapatra, S., Chakraborty, T., Kumar, V., DeBoeck, G., & Mohanta, K. N. (2013). Aquaculture and stress management: a review of probiotic intervention. *Journal of animal physiology and animal nutrition*, *97*(3), 405-430

Mohapatra, S., Chakraborty, T., Prusty, A. K., Das, P., Paniprasad, K., & Mohanta, K. N. (2012). Use of different microbial probiotics in the diet of rohu, Labeo rohita fingerlings: effects on growth, nutrient digestibility and retention, digestive enzyme activities and intestinal microflora. *Aquaculture Nutrition*, *18*(1), 1-11.

Moriarty, W. J. D., Decamp, O., & Lavens, P. (2005). Probiotics in aquaculture. AQUA Culture Asia Pacific Magazine. September.

Mulder, I. E., Schmidt, B., Stokes, C. R., Lewis, M., Bailey, M., Aminov, R. I., ... & Musk, C. C. (2009). Environmentally-acquired bacteria

influence microbial diversity and natural innate immune responses at gut surfaces. *BMC biology*, *7*(1), 79.

Naser, M. E., El-Motaal, A., Sabry, M., El-Sheikh, S. M., & Mahmoud, S. H. (2019). Effects of commercial and fish isolated gut Bacillus strains supplementation on the performance of Oreochromis niloticus challenged with Aeromonas hydrophila. *Egyptian Journal for Aquaculture*, *9*(1), 61-75.

Nayak, S. K. (2010 b). Probiotics and immunity: a fish perspective. *Fish & shellfish immunology*, *29*(1), 2-14.

Nayak, S. K. (2010a). Role of gastrointestinal microbiota in fish. *Aquaculture Research*, *41*(11), 1553-1573.

Ni, J., Yan, Q., Yu, Y., & Zhang, T. (2014). Factors influencing the grass carp gut microbiome and its effect on metabolism. *FEMS Microbiology Ecology*, *87*(3), 704-714.

Nicholson, W. L. (2004). Ubiquity, longevity, and ecological roles of Bacillus spores. *Bacterial spore formers: probiotics and emerging applications*, 1-15.

Nielsen, D. S., Cho, G. S., Hanak, A., Huch, M., Franz, C. M., & Arneborg, N. (2010). The effect of bacteriocin-producing Lactobacillus plantarum strains on the intracellular pH of sessile and planktonic Listeria monocytogenes single cells. *International journal of food microbiology*, *141*, S53-S59.

Nikoskelainen, S., Ouwehand, A. C., Bylund, G., Salminen, S., & Lilius, E. M. (2003). Immune enhancement in rainbow trout (Oncorhynchus mykiss) by potential probiotic bacteria (Lactobacillus rhamnosus). *Fish & shellfish immunology*, *15*(5), 443-452.

Nurmi, J. T., Puolakkainen, P. A., & Rautonen, N. E. (2005). Bifidobacterium Lactis sp. 420 up-regulates cyclooxygenase (Cox)-1 and

down-regulates Cox-2 gene expression in a Caco-2 cell culture model. *Nutrition and cancer*, *51*(1), 83-92.

Oelschlaeger, T. A. (2010). Mechanisms of probiotic actions–a review. *International Journal of Medical Microbiology*, *300*(1), 57-62.

Oliveira, R. V., Peixoto, P. G., Ribeiro, D. D. C., Araujo, M. C., do Santos, C. T. B., Hayashi, C., & Pelli, A. (2014). Klebsiella pneumoniae as a main cause of infection in Nishikigoi Cyprinus carpio (carp) by inadequate handling. *Brazilian Journal of Veterinary Pathology*, *7*(2), 86-88.

Olmos, J., & Paniagua-Michel, J. (2014). Bacillus subtilis a potential probiotic bacterium to formulate functional feeds for aquaculture. *J. Microb. Biochem. Technol*, *6*(7), 361-365.

Olmos, J., Ochoa, L., Paniagua-Michel, J., & Contreras, R. (2011). Functional feed assessment on Litopenaeus vannamei using 100% fish meal replacement by soybean meal, high levels of complex carbohydrates and Bacillus probiotic strains. *Marine drugs*, *9*(6), 1119-1132.

O'Shea, E. F., Cotter, P. D., Stanton, C., Ross, R. P., & Hill, C. (2012). Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: bacteriocins and conjugated linoleic acid. *International journal of food microbiology*, *152*(3), 189-205.

Paniagua-Michel, J., Franco-Rivera, A., Cantera, J. J. L., & Stein, L. Y. (2005). Activity of nitrifying biofilms constructed on low-density polyester enhances bioremediation of a coastal wastewater effluent. *World Journal of Microbiology and Biotechnology*, *21*(8-9), 1371-1377.

Panigrahi, A., & Azad, I. S. (2007). Microbial intervention for better fish health in aquaculture: the Indian scenario. *Fish physiology and biochemistry*, *33*(4), 429-440.

Panigrahi, A., Kiron, V., Satoh, S., & Watanabe, T. (2010). Probiotic bacteria Lactobacillus rhamnosus influences the blood profile in rainbow trout Oncorhynchus mykiss (Walbaum). *Fish physiology and biochemistry*, *36*(4), 969-977.

Panigrahi, A., Kiron, V., Satoh, S., Hirono, I., Kobayashi, T., Sugita, H., ... & Aoki, T. (2007). Immune modulation and expression of cytokine genes in rainbow trout Oncorhynchus mykiss upon probiotic feeding. *Developmental & Comparative Immunology*, *31*(4), 372-382.

Parkos, J. I. I. I., & Wahl, D. (2014). Effects of common carp (Cyprinus carpio), an exotic fish, on Aquatic Ecosystems. Ilinois Natural History Survey report of January/February 2000. University of Illinois Board of Trustees. *Center for Aquatic Ecology*.

Perez-Sanchez T, Balcazar JL, Garcı´a Y, Halaihel N, Vendrell D, DeBlas I, Merrifield DL, Ruiz-Zarzuela I (2011) Identification and characterization of lactic acid bacteria isolatedfrom rainbow trout (Oncorhynchus mykiss) withinhibitory activity against Lactococcus garvieae. *Journal Fish Dis*34*:*499–507.

Perdigon, G., Galdeano, C. M., Valdez, J. C., & Medici, M. (2002). Interaction of lactic acid bacteria with the gut immune system. *European journal of clinical nutrition*, *56*(4), S21-S26.

Pieters, N., Brunt, J., Austin, B., & Lyndon, A. R. (2008). Efficacy of in-feed probiotics against Aeromonas bestiarum and Ichthyophthirius multifiliis skin infections in rainbow trout (Oncorhynchus mykiss, Walbaum). *Journal of Applied Microbiology*, *105*(3), 723-732.

Pirarat, N., Kobayashi, T., Katagiri, T., Maita, M., & Endo, M. (2006). Protective effects and mechanisms of a probiotic bacterium Lactobacillus rhamnosus against experimental Edwardsiella tarda infection in tilapia

(Oreochromis niloticus). *Veterinary immunology and immunopathology*, *113*(3-4), 339-347.

Pommerville, J. C. (2007). *Alcamo's laboratory fundamentals of microbiology*. Jones & Bartlett Learning.

Prakash, S., Rodes, L., Coussa-Charley, M., & Tomaro-Duchesneau, C. (2011). Gut microbiota: next frontier in understanding human health and development of biotherapeutics. *Biologics: targets & therapy*, *5*, 71.

Pressley, M. E., Phelan III, P. E., Witten, P. E., Mellon, M. T., & Kim, C. H. (2005). Pathogenesis and inflammatory response to Edwardsiella tarda infection in the zebrafish. *Developmental & Comparative Immunology*, *29*(6), 501-513.

Rahman, M. M. (2015). Role of common carp (Cyprinus carpio) in aquaculture production systems. *Frontiers in Life Science*, *8*(4), 399-410.

Rahman, M. M., Noor, N. M., Saad, S., & Yunus, K. (2016). Coastal water quality of Tioman Island: effects of human activity and the distance from shoreline. *Desalination and Water Treatment*, *57*(1), 83-87.

Ramos, M. A., Weber, B., Gonçalves, J. F., Santos, G. A., Rema, P., & Ozório, R. O. A. (2013). Dietary probiotic supplementation modulated gut microbiota and improved growth of juvenile rainbow trout (Oncorhynchus mykiss). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *166*(2), 302-307.

Rawls, J. F., Samuel, B. S., & Gordon, J. I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences*, *101*(13), 4596-4601.

Ray, A. K., Ghosh, K., & Ringø, E. J. A. N. (2012). Enzyme-producing bacteria isolated from fish gut: a review. *Aquaculture Nutrition*, *18*(5), 465-492.

Ringø, E., Løvmo, L., Kristiansen, M., Bakken, Y., Salinas, I., Myklebust, R., ... & Mayhew, T. M. (2010). Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a review. *Aquaculture Research*, *41*(4), 451-467

Ringø, E., Olsen, R. E., Mayhew, T. M., & Myklebust, R. (2003). Electron microscopy of the intestinal microflora of fish. *Aquaculture*, *227*(1-4), 395-415.

Ringø, E., Sperstad, S., Myklebust, R., Refstie, S., & Krogdahl, Å. (2006). Characterisation of the microbiota associated with intestine of Atlantic cod (Gadus morhua L.): the effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture*, *261*(3), 829-841.

Ringø, E., Zhou, Z., Vecino, J. G., Wadsworth, S., Romero, J., Krogdahl, Å., ... & Owen, M. (2016). Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story?. *Aquaculture nutrition*, *22*(2), 219-282.

Robertson, P. A. W., O'Dowd, C., Burrells, C., Williams, P., & Austin, B. (2000). Use of Carnobacterium sp. as a probiotic for Atlantic salmon (Salmo salar L.) and rainbow trout (Oncorhynchus mykiss, Walbaum). *Aquaculture*, *185*(3-4), 235-243.

Rollo, A., Sulpizio, R., Nardi, M., Silvi, S., Orpianesi, C., Caggiano, M., ... & Carnevali, O. (2006). Live microbial feed supplement in aquaculture

for improvement of stress tolerance. *Fish Physiology and Biochemistry*, *32*(2), 167-177.

Russo, P., Arena, M. P., Fiocco, D., Capozzi, V., Drider, D., & Spano, G. (2017). Lactobacillus plantarum with broad antifungal activity: A promising approach to increase safety and shelf-life of cereal-based products. *International journal of food microbiology*, *247*, 48-54.

Rutherfurd-Markwick, K. J., & Gill, H. S. (2004). Probiotics and immunomodulation. In *Diet and human immune function* (pp. 327-344). Humana Press, Totowa, NJ.

Sallusto, F., & Lanzavecchia, A. (2009). Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. *European journal of immunology*, *39*(8), 2076-2082.

Salyers, A. A., & Whitt, D. D. (2002). How bacteria become resistant to antibiotics. *Bacterial Pathogenesis: A Molecular Approach. American Society for Microbiology Washington DC*, 168-184.

Sampson, T. R., & Mazmanian, S. K. (2015). Control of brain development, function, and behavior by the microbiome. *Cell host & microbe*, *17*(5), 565-576.

Satheeshkumar, P., Ananthan, G., Kumar, D. S., & Jagadeesan, L. (2011). Haematology and biochemical parameters of different feeding behaviour of teleost fishes from Vellar estuary, India. *Comparative Clinical Pathology*, *21*(6), 1187-1191.

Saxena, V., Lienesch, D. W., Zhou, M., Bommireddy, R., Azhar, M., Doetschman, T., & Singh, R. R. (2008). Dual roles of immunoregulatory

cytokine TGF-β in the pathogenesis of autoimmunity-mediated organ damage. *The Journal of Immunology*, *180*(3), 1903-1912.

Schrezenmeir, J., & de Vrese, M. (2001). Probiotics, prebiotics, and synbiotics—approaching a definition. *The American journal of clinical nutrition*, *73*(2), 361s-364s.

Secombes, C. J., & Wang, T. (2012). The innate and adaptive immune system of fish. In *Infectious disease in aquaculture* (pp. 3-68). Woodhead Publishing.

Semova, I., Carten, J. D., Stombaugh, J., Mackey, L. C., Knight, R., Farber, S. A., & Rawls, J. F. (2012). Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. *Cell host & microbe*, *12*(3), 277-288.

Senok, A. C., Ismaeel, A. Y., & Botta, G. A. (2005). Probiotics: facts and myths. *Clinical Microbiology and Infection*, *11*(12), 958-966

Shaio, M.-F., LIN, P.-R. & Liu, J.-Y. 1997. Colorimetric one-tube nested PCR for detection of *Trichomonas vaginalis* in vaginal discharge. *Journal of Clinical Microbiology,* 35**,** 132-138.

Sharifuzzaman, S. M., Al-Harbi, A. H., & Austin, B. (2014). Characteristics of growth, digestive system functionality, and stress factors of rainbow trout fed probiotics Kocuria SM1 and Rhodococcus SM2. *Aquaculture*, *418*, 55-6

Sharma, P., Sihag, R. C., & Gahlawat, S. K. (2013). Relative efficacy of two probiotics in controlling the epizootic ulcerative syndrome disease in Mrigal (Cirrhinus mrigala Ham.). *Journal of Fisheries and Aquatic Science*, *8*(2), 305.

Sheng, Y., Ren, H., Limbu, S. M., Sun, Y., Qiao, F., Zhai, W., ... & Zhang, M. (2018). The presence or absence of intestinal microbiota affects lipid deposition and related genes expression in zebrafish (Danio rerio). *Frontiers in microbiology*, *9*, 112.

Shin-ichiro, S. M., Usio, N., Takamura, N., & Washitani, I. (2009). Contrasting impacts of invasive engineers on freshwater ecosystems: an experiment and meta-analysis. *Oecologia*, *158*(4), 673-686.

Silbergeld, E. K. (1974). Blood glucose: a sensitive indicator of environmental stress in fish. *Bulletin of Environmental Contamination and Toxicology*, *11*(1), 20-25.

Singh, V. P. (2018). Recent approaches in food bio-preservation-a review. *Open veterinary journal*, *8*(1), 104-111.

Soltani, M., Sheikhzadeh, N., Ebrahimzadeh-Mousavi, H. A., & Zargar, A. (2010). Effects of Zataria multiflora essential oil on innate immune responses of common carp (Cyprinus carpio). *Journal of Fisheries and Aquatic Science*, *5*(3), 191-199.

Sonenshein, A. L., Hoch, J. A., & Losick, R. (1993). *Bacillus subtilis and other gram-positive bacteria*. American Society for Microbiology.

Song, S. K., Beck, B. R., Kim, D., Park, J., Kim, J., Kim, H. D., & Ringø, E. (2014). Prebiotics as immunostimulants in aquaculture: a review. *Fish & shellfish immunology*, *40*(1), 40-48.

Sorokulova, I. (2013). Modern status and perspectives of Bacillus bacteria as probiotics. *J Prob Health*, *1*(4), e106.

Sørum, H. (2006). Antimicrobial drug resistance in fish pathogens. *Antimicrobial resistance in bacteria of animal origin*, 213-238.

Standen, B. T., Peggs, D. L., Rawling, M. D., Foey, A., Davies, S. J., Santos, G. A., & Merrifield, D. L. (2016). Dietary administration of a commercial mixed-species probiotic improves growth performance and modulates the intestinal immunity of tilapia, Oreochromis niloticus. *Fish & Shellfish Immunology*, *49*, 427-435.

Suva, M. A., Sureja, V. P., & Kheni, D. B. (2016). Novel insight on probiotic Bacillus subtilis: mechanism of action and clinical applications. *Journal of Current Research in Scientific Medicine*, *2*(2), 65.

Tafalla, C., Coll, J., & Secombes, C. J. (2005). Expression of genes related to the early immune response in rainbow trout (Oncorhynchus mykiss) after viral haemorrhagic septicemia virus (VHSV) infection. *Developmental & Comparative Immunology*, *29*(7), 615-626.

Tang ,Y. W., & Stratton, C. W. (2006). Advanced Techniques in Diagnostic Microbiology. Springer Science+ Business Media, LLC. *Printed in the United States of America technology, 47,* 309-317.

Tassakka, A. C. M. A., & Sakai, M. (2004). Expression of immune-related genes in the common carp (Cyprinus carpio L.) after stimulation by CpG oligodeoxynucleotides. *Aquaculture*, *242*(1-4), 1-12.

Tinh, N. T. N., Dierckens, K., Sorgeloos, P., & Bossier, P. (2008). A review of the functionality of probiotics in the larviculture food chain. *Marine Biotechnology*, *10*(1), 1-12.

Toranzo, A. E., Devesa, S., Romalde, J. L., Lamas, J., Riaza, A., Leiro, J., & Barja, J. L. (1995). Efficacy of intraperitoneal and immersion vaccination against Enterococcus sp. infection in turbot. *Aquaculture*, *134*(1-2), 17-27.

Tukmechi, A., Morshedi, A., & Delirezh, N. (2007). Changes in intestinal microflora and humoral immune response following probiotic administration in rainbow trout (Oncorhynchus mykiss).

Uddin, N., & Al-Harbi, A. H. (2012). Bacterial flora of polycultured common carp (Cyprinus carpio) and African catfish (Clarias gariepinus). *International Aquatic Research*, *4*(1), 10.

Valdez, A., Yepiz-Plascencia, G., Ricca, E., & Olmos, J. (2014). First L itopenaeus vannamei WSSV 100% oral vaccination protection using C ot C:: V p26 fusion protein displayed on B acillus subtilis spores surface. *Journal of applied microbiology*, *117*(2), 347-357.

Venkateswara, A. R. (2007). Bioremediation to restore the health of aquaculture. *Pond Ecosystem Hyderabad*, *500*(082), 1-12.

Verschuere, L., Rombaut, G., Sorgeloos, P., & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.*, *64*(4), 655-671.

Vivas, J., Riaño, J., Carracedo, B., Razquin, B. E., López-Fierro, P., Naharro, G., & Villena, A. J. (2004). The auxotrophic aroA mutant of Aeromonas hydrophila as a live attenuated vaccine against A. salmonicida infections in rainbow trout (Oncorhynchus mykiss). *Fish & Shellfish Immunology*, *16*(2), 193-206.

Vreeland, R. H., Rosenzweig, W. D., & Powers, D. W. (2000). Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. *Nature*, *407*(6806), 897-900.

Wan, Y. Y., & Flavell, R. A. (2007). 'Yin–Yang'functions of transforming growth factor-β and T regulatory cells in immune regulation. *Immunological reviews*, *220*(1), 199-213

Wang, L., Ge, C., Wang, J., Dai, J., Zhang, P., & Li, Y. (2017). Effects of different combinations of Bacillus on immunity and antioxidant activities in common carp. *Aquaculture International*, *25*(6), 2091-2099.

Wang, T., & Secombes, C. J. (2015). The evolution of IL-4 and IL-13 and their receptor subunits. *Cytokine*, *75*(1), 8-13.

Wang, Y. B., Tian, Z. Q., Yao, J. T., & Li, W. F. (2008). Effect of probiotics, Enteroccus faecium, on tilapia (Oreochromis niloticus) growth performance and immune response. *Aquaculture*, *277*(3-4), 203-207.

Wang, Y. C., Chang, P. S., & Chen, H. Y. (2008). Differential time-series expression of immune-related genes of Pacific white shrimp Litopenaeus vannamei in response to dietary inclusion of β-1, 3-glucan. *Fish & Shellfish Immunology*, *24*(1), 113-121.

Weber, M. J., & Brown, M. L. (2011). Relationships among invasive common carp, native fishes and physicochemical characteristics in upper Midwest (USA) lakes. *Ecology of Freshwater Fish*, *20*(2), 270-278

Woynarovich, A., Moth-Poulsen, T., & Péteri, A. (2010). *Carp polyculture in Central and Eastern Europe, the Caucasus and Central Asia: a manual* (Vol. 554). Food and Agriculture Organization of the United Nations.

Xiaoyun, Z., Mingyun, L., Khalid, A., & Weinmin, W. (2009). Comparative of haematology and serum biochemistry of cultured and wild Dojo loach Misgurnus anguillicadatus. *Fish Physiol Biochem*, *35*, 435-441 .

Yang, M., Wang, Y., Wang, X., Chen, C., & Zhou, H. (2010). Characterization of grass carp (Ctenopharyngodon idellus) Foxp1a/1b/2: evidence for their involvement in the activation of peripheral blood lymphocyte subpopulations. *Fish & shellfish immunology*, *28*(2), 289-295.

Yassir, A. L., Adel, M. E., & Azze, A. (2002). Use of probiology bacteria as growth promoters, antibacterial and the effect on physiological parameters of Oreochronus niroticus. *J Fish Dis*, *25*, 633-642.

Zhang, L., Yi, H., Xia, X. P., & Zhao, Y. (2006). Transforming growth factor-beta: an important role in CD4+ CD25+ regulatory T cells and immune tolerance. *Autoimmunity*, *39*(4), 269-276.

Zorriehzahra, M. J., Delshad, S. T., Adel, M., Tiwari, R., Karthik, K., Dhama, K., & Lazado, C. C. (2016). Probiotics as beneficial microbes in aquaculture: an update on their multiple modes of action: a review. *Veterinary quarterly*, *36*(4), 228-241.

الخلاصة

أجريت الدراسة الحالية للكشف تأثير المكملات الغذائية لبكتريا ال *Bacillus subtilis* على المجتمع الميكروبي المعوي، وأداء النمو ومؤشرات الدم ، ومؤشرات الكيمياء الحيوية في المصل ومستويات السايتوكينات بما في ذلك (*TGFβ* و*IL1β*) في أسماك الكارب

جمعت سبعون سمكة بمعدل اوزان (77.2 ± 0.86 g) , ووزعت بشكل عشوائي في اربعة عشر حوض بلاستيكي . سبعة احواض سيطرة وسبعة احواض بروبايوتك كل حوض يحتوي على خمس اسماك .تم اطعام الأسماك بنسبة 1.5-2% من الوزن الاولي لها يوميا .تم عزل بكتريا ال *B.subtilis* من التربة الموقعية وتم تشخيصها مختبريا بشكل أولي ثم تم تأكيد التشخيص باستخدام تقنية تفاعل البلمرة المتسلسل لتضخيم الجين. 16S *rDNA* .

تم تغذية الأسماك بعليقة تجارية تحتوي على البكتريا بتركيز (10^7 CFU g^{-1}~ و عليقة بدون إضافة البكتريا كمجموعة للسيطرة لمدة ستة أسابيع .تم حساب العدد الحي الكلي للبكتريا باستخدام وسط تربتون صويا اكار بينما تم حساب بكتريا حامض اللبنيك باستخدام وسط de Man, Rogosa and Sharpe agar.

اثبتت نتائج الفحص الميكروبي بأن العدد الكلي للبكتريا الحية وبكتريا حامض اللبن تناقص بشكل معنوي في مجموعة الأسماك التي غذيت عليقة البكتريا بالمقارنة مع مجموعة السيطرة بينما ازداد المجتمع الميكروبي الغير متأصلة يعني الموجودة في عينات خروج الأسماك للعدد الكلي البكتيري الحي وبشكل معنوي في مجموعة الأسماك مع بكتريا البروبيوتك بالمقارنة مع مجموع السيطرة.

تم تصنيف مجتمع الاحياء المجهرية لأسماك الكارب من خلال الفحص البكتيري الى نوعين من العوائل وهي Proteobacteria and Firmicutes

بينما اشارت النتائج الى عدم تأثر مؤشرات النمو بالبكتريا .بالمقارنة بمجموعة اسماك السيطرة أظهرت الأسماك التي تمت تغذيتها على العليقة مع البكتريا زيادة معنوية في تركيزلزوجة الهيموكلوبين الجسيمي .($P < 0.05$) بينما لم تتأثر مؤشرات الدم الأخرى معنويا مثل اليوريا والكوليسترول وتركيز السكر في الدم بالبكتريا .ومع ذلك فان تراكيز مرتفعة معنوية للكرياتنين لوحظت بتأثير بكتريا ال *B. Subtilis*عنها في مجموعة السيطرة.

أظهرت نتائج فحص الاليزا تراكيز معنوية مرتفعة لل IL1β في مجموعة اسماك المعاملة بالمقارنة مع اسماك السيطرة (P=0.004) بينما وجدت تراكيز معنوية منخفضة من TGFβ في مجموعة اسماك المعاملة بالمقارنة مع اسماك السيطرة (P=0.05) .

النتائج المتحصلة من الدراسة الحالية أوضحت دور محتمل لبكتريا *B. subtilis* في تحوير المجتمع البكتيري للأمعاء وكذلك الحالة الصحية لأسماك الكارب .

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تاثير بكتريا(*Bacillus subtilis*)على المجتمع المايكروبي ودلائل تحسين النموو المؤشرات الدموية والمناعية لأسماك الكارب (*Cyprinus carpio*)

رسالة مقدمة الى

مجلس كلية العلوم / جامعة كربلاء

وهي جزء من متطلبات نيل شهادة الماجستير في العلوم / علوم الحياة

من قبل الطالبة

دعاء علي بعيوي الغانمي

(بكالوريوس علوم / علوم الحياة / جامعة القاد سية ـ٢٠٠٩)

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