IN VITRO AND IN VIVO STUDIES OF CHICKEN GUT TO PRODUCE CHICKEN PROBIOTICS BY ISOLATED MICROBES FROM LOCAL FERMENTED FOODS

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IN VITRO AND IN VIVO STUDIES OF CHICKEN GUT TO PRODUCE CHICKEN PROBIOTICS BY ISOLATED MICROBES FROM LOCAL FERMENTED FOODS

by

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DECLARATION

I hereby declare that this dissertation, submitted to Universiti Sains Malaysia as a fulfilment of the requirement for the Master of Science degree, has not been submitted to any other university for any degree. I also certify that the work described herein is entirely my own, except as cited in as references and summaries sources of which have been duly acknowledged.

SHAKERA JAKERA

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%	Percentage
μ	Specific growth rate
T_d	Doubling time
°C	Degree Celsius

LIST OF ABBREVIATIONS

GIT	Gastrointestinal tract
MRS	de Mann Rogosa and Sharpe
SD	Sabouraud Dextrose
OD	Optical density
nm	Nanometer
hr	Hour
µm	Micrometer
M	Molar
mL	Milliliter
SY	Sample Yeast
SB	Sample Bacteria
g/ml	Gram per milliliter
GCMS	Gas Chromatography Mass Spectrometry
AGP	Antibiotic Growth Promoter

LIST OF APPENDICES

KAJIAN 'IN VITRO' DAN 'IN VIVO' KE ATAS USUS AYAM UNTUK MENGHASILKAN PROBIOTIK AYAM MELALUI PEMENCILAN MIKROB DARI MAKANAN TEMPATAN TERTAPAI

ABSTRAK

Di peringkat global, penggunaan makanan ayam semakin meningkat kerana keperluan dan pengeluaran daging dan telur ayam yang konsisten. Telur dan daging membekalkan kita protein, iodin, zat besi, zink, mineral dan vitamin. Beberapa agen antimikrob dan antibiotik yang disintesis secara kimia kini digunakan dalam makanan ayam sebagai penggalak pertumbuhan dan untuk meningkatkan prestasi. Sisa antibiotik ini akan kekal dalam daging ayam, tulang, dan organ dan ia boleh memberi kesan negatif kepada kesihatan manusia. Probiotik adalah satu penyelesaian, namun tidak semua probiotik yang diisolasi dan dihasilkan berguna untuk ayam itik dan bertindak dengan baik. Kek soya dan pes udang yang ditapai telah digunakan dalam kajian ini untuk mengasingkan potensi probiotik ayam seperti bakteria dan yis. Bagi kajian ini, terdapat pengasingan mikroorganisma daripada pes udang yang ditapai dan kuih soya, sembilan yis dan sepuluh bakteria telah dipilih sebagai sampel ujian. Selepas mengisolasikan organisma, bagi setiap mikroorganisma, analisis in vitro dijalankan sambil mengekalkan parameter usus ayam penuh, yang merangkumi suhu 37°C, keadaan anaerobik dan tujuh pH berbeza (2.5, 3.5, 5, 6.5, 7, 7.5, dan 8). Berikutan ujian in vitro, strain SB3, SB5, dan SB10 mengatasi strain lain dari segi kadar pertumbuhan dan pengeluaran biojisim merentas semua julat pH. Pada semua tahap pH, SY1, SY2, SY3, SY4 dan SY8 meningkat lebih tinggi dan menghasilkan lebih banyak. Strain mikrob akhir ditentukan dengan menilai semua data, termasuk

penemuan GCMS, dan ditentukan sebagai SB10 dan SY3. Mikroorganisma telah dikenal pasti melalui analisis rRNA 16S sebagai *Staphylococcus hominis* dan *Cutaneotrichosporon arboriforme* (*C. arboriforme*). Selepas itu, bahagian in vivo dilakukan dalam dua peringkat. Peringkat pertama dimulakan dengan enam ekor anak ayam. Dalam tempoh pemantauan 40 hari, berat badan mereka, sisa makanan ditimbang setiap hari, air bersih yang bersih diberikan, dan persekitaran yang bersih dikekalkan dengan ketat. Dua daripadanya diberi sampel bakteria (ayam B), dua daripadanya diberi sampel yis (ayam Y) setiap dua hari untuk mengekalkan pertumbuhan mikrob usus mereka, dan dua lagi ayam dikekalkan secara normal (ayam C). Anak ayam ragi menggunakan lebih banyak makanan daripada dua kelompok yang lain dan membesar dengan terbaik, manakala ayam B berkembang menjadi yang kedua terbaik. Ayam yang dirawat dengan dua sampel probiotik mempunyai FCR yang lebih besar dari awal. Bahagian in vivo dilakukan, diikuti dengan peringkat ke-2, iaitu analisis kandungan lembapan (64% hingga 72%) tahi ayam dan daging serta hati ayam Y mengandungi protein paling banyak (86.04% dan 46.47%, masing-masing), manakala gizzard ayam B mengandungi protein paling banyak (34.22%). Daging dan hempedu ayam kawalan mempunyai paling banyak lipid (11.67% dan 35.67%). Berat hati ayam nampaknya lebih tinggi untuk ayam yang dirawat dengan probiotik, ayam Y 48.25 gm, ayam B 42.04 gm dan ayam C 30.18 gm. Berat ayam gizzard nampaknya kurang untuk ayam yang dirawat dengan probiotik ayam Y 18.32 gm, ayam B 16.75 gm dan ayam C 23.18 gm. Kesimpulannya, mikroorganisma yang diisolasi daripada makanan tempatan yang ditapai berpotensi sebagai probiotik ayam berdasarkan pertumbuhannya dalam persekitaran usus ayam. Industri ayam akan mendapat manfaat daripada kajian meluas ini, dan penyelidikan ayam akan berkembang.

IN VITRO AND IN VIVO STUDIES OF CHICKEN GUT TO PRODUCE CHICKEN PROBIOTICS BY ISOLATED MICROBES FROM LOCAL FERMENTED FOODS

ABSTRACT

Globally, the use of poultry feed is increasing because of the consistent needs and production of poultry meat and eggs. Egg and meat provide us proteins, iodine, iron, zinc, minerals, and vitamins. Several chemically synthesized antimicrobial agents and antibiotics are currently used in poultry feed as growth promoters and to improve performance. These antibiotic residues will remain in chicken meat, bones, and organs and may eventually have a negative impact on human health. Probiotics are one solution, however not all isolated and produced probiotics are useful for poultry and act well. Fermented soy cake and shrimp paste was used in this study to isolate potential chicken probiotic such as bacteria and yeast. For this study, including the isolation of microorganisms from fermented shrimp paste and soya cake, nine yeast and ten bacteria were selected as test sample. After isolating the organisms, for each microorganism, an in vitro analysis was conducted while maintaining full chicken gut parameters, which includes 37°c temperature, anaerobic condition and seven different pH (2.5, 3.5, 5, 6.5, 7, 7.5, and 8). Following in vitro testing, SB3, SB5, and SB10 strains outgrew other strains in terms of growth rate and biomass production across all pH ranges. At all pH levels, SY1, SY2, SY3, SY4, and SY8 grew higher and yielded more. The final microbial strain was determined by evaluating all of the data, including the GCMS findings, and was determined to be SB10 and SY3. The microorganisms were identified by 16S rRNA analysis as *Staphylococcus hominis* and *Cutaneotrichosporon arboriforme* (*C. arboriforme*).

After that, the in vivo part was done in two stages. The first stage started with six chicks. In the 40-day monitoring period, their weight, left-over feeds were weighted daily, clean fresh water was given, and a clean environment was maintained strictly. Two of them were fed bacteria samples (B chicken), two of them were fed yeast samples (Y chicken) every two days to maintain their gut microbe's growth, and the other two chickens were maintained normally (C chicken). Yeast chicks consumed more feed than the other two batches and grew the best, while B chickens grew the second best. The chickens treated with two probiotic samples had a greater FCR from the beginning. The in vivo part was done, followed by the 2^{nd} stage, which was an analysis of the moisture content (64% to 72%) of the chicken manure and the meat and liver of Y chickens contain the most protein (86.04% and 46.47%, respectively), while the gizzard of B chickens contains the most protein (34.22%). The meat and gizzard of control chickens have the most lipids (11.67% and 35.67%). Weight of chicken liver seems higher for the chickens which were treated with probiotic, Y chicken 48.25 gm, B chicken 42.04 gm and C chicken 30.18 gm. Chicken gizzard weight seems to less for the chickens which were treated with probiotics Y chicken 18.32 gm, B chicken 16.75 gm and C chicken 23.18 gm. In conclusion, isolated microorganisms from fermented local foods have a potential as a chicken probiotic based on their growth in the chicken gut environment. The poultry industry will benefit from this extensive study, and poultry research will progress.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Chicken meat production was estimated to reach 137 million tonnes by 2020 and became the most popular animal meat in the world [1]. It becomes the primary protein source, and it is less expensive than red meat. Chicken flesh is high in amino acids, minerals, vitamins, choline, zinc, iron, copper, and other nutrients, and chicken meat also contains tryptophan; an amino acid related to higher amounts of serotonin known as the "feel good" hormone in human brains[2]. Red meats, on average, have more saturated fat than chicken meat. Saturated fats elevate blood cholesterol and put human at risk for heart disease[3]. Chicken is an excellent red meat replacement. Meat has been connected to a range of health benefits in addition to being a fantastic source of protein [2]. Antibiotics are used in the chicken industry to boost meat output by increasing feed conversion, promoting growth rate, and preventing disease [4]. Antibiotics as growth promoters also known as AGP, have been utilised in the broiler industry for more than five decades in various countries [5]. Antibiotic resistance in chicken can result from the unrestricted use of antibiotics as AGP, as well as pre-colonized antibiotic-resistant bacteria previously present in the population [6]. Resistance to antibiotics is one of the concerns that the poultry industry is dealing with, and it has resulted in a high illness and fatality among chickens during various disease outbreaks. Through direct contact, food-produced animals, or indirectly via atmospheric channels, antibiotic resistance can be passed on to other animals or people [7]. Antibiotic resistance occurs when microorganisms gain the capacity to overcome antibiotics that were intended to kill them [8]. Antibiotic resistance can

harm human health at any age, and the healthcare, animal, and farming industries, as a result, this is one of the world's most challenging public health concerns [8]. Antibiotics have been found to enhance the prevalence of gut infections and the host's sensitivity to them [9][10]. The use of oral antibiotics to alter the intestinal microbiome of chickens throughout their early lives has been linked to immunological dysfunction, including the development of autoimmune disorders [10]. The demand to reduce the use of AGP in poultry is increasing and becoming irreversible, and numerous nations are enforcing restrictions and bans on AGP use, the European Union placed an absolute prohibition on all AGP in 2006 [11]. The restriction on antibiotics in broiler chicken feed, combined with rising customer preferences for antibiotic-free poultry products, has piqued the attention of poultry scientists and manufacturers in finding viable antibiotic replacements in recent decades [11]. AGP has been replaced with a variety of feed additives in chicken, with variable degrees of success [12]. Chickens with probiotics have a higher feed conversion ratio [13]. Chickens that consume probiotics can stay at a healthy weight and produce high-quality eggs by maintaining a healthy digestive tract [13]. Beyond offering fundamental nutritional benefits, probiotics provide a long list of health advantages. In commercial broilers, probiotic supplements containing beneficial microflora can aid in the establishment and maintenance of intestinal microbiota balance [14]. It is already well accepted that keeping a healthy microbiota gives a variety of nutritional benefits, as well as intestinal mucosa development, pathogen protection, and immunological maturation [15]. However, the focus of this research was on the isolation, development, in vitro, and in vivo testing of potential probiotics isolated from locally fermented foods.

1.2 Problem statements

The poultry sector is now dealing with a slew of issues, including antibiotic contamination of chicken products. Antibiotics were added into poultry feed or drinks to encourage rapid growth. Some antibiotic residues will, however, persist in the internal organs or meat. After consuming the chicken products, the antibiotic residue can remain in the human body. Many methods have recently been developed to minimize or limit the use of antibiotics, such as the use of probiotics and vaccinations in the chicken industry. This study explores the influence of isolated microorganisms on chicken development by using them as probiotics.

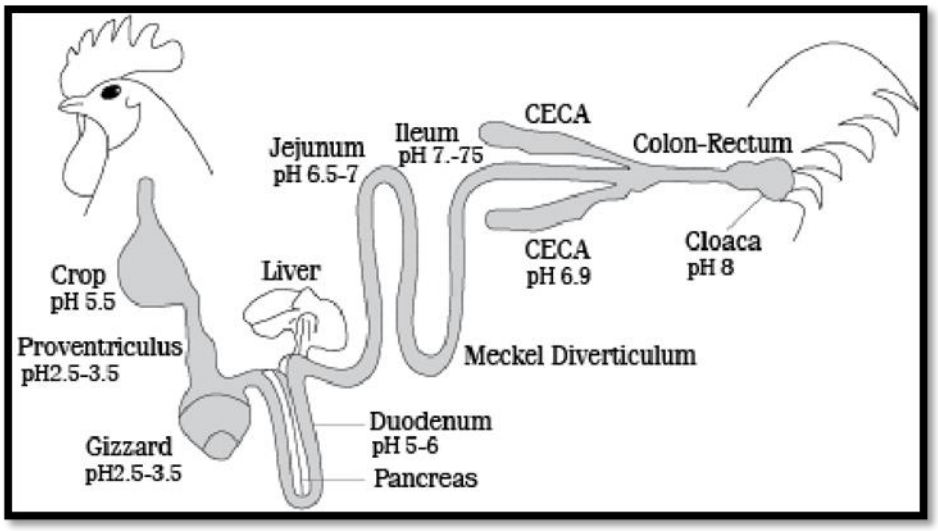

Figure 1.1: Different pH of chicken gastrointestinal tract (GIT) [16]

Most fermented foods are known to contain probiotics. Various forms of probiotics are beneficial for specific living beings, and their effectiveness is determined by elements in the environment of the living being's gut or digestive system. The gastrointestinal tract of chickens has a pH range that varies from very acidic to basic [17]. Figure 1.1 shows the different pH balance of chicken gastrointestinal tract (GIT).

There are multiple kinds of probiotics, however it has yet to be established that they can survive in all pH levels of the chicken GIT. This study used in vitro testing to find bacteria that can thrive in a variety of pH environments. Following AGP's removal, the industry has experienced decreased production efficiency, bacterial overgrowth in the intestine, nutrient loss, and food contamination [18]. As antibiotics are phased out, it must be established that chicken supplemented with probiotics provides improved health, better food conversion efficiency, and other benefits as better protein and less lipid ratio for a healthy diet. Improved health, better food conversion ratio, and other positive results were seen in the in vivo monitoring and analysis

1.3 Research objectives

The objectives of this research are shown below:

1. To isolate and investigate selected microorganisms from local fermented foods such as fermented shrimp paste and soya cake for further in-vitro studies of chicken guts.
2. To identify potential microorganisms from isolated yeast and bacteria that can survive in the chicken gut under diverse pH and environmental conditions.
3. To investigate the effects of isolated and selected yeast and bacteria on chicken growth rate, manure, meat, liver, and gizzard analyses throughout a 40-day in-vivo investigation.

1.4 Originality of research

The originality of this research, as mentioned below:

1) Microbes were isolated from local Southeast Asian fermented foods such soy cake and shrimp paste to develop probiotics for broilers.

2) In-vitro and in-vivo experiments were executed to evaluate the probiotics' viability and survival in the chicken gut. In-vitro experiment was performed to determine how many of the isolated microbes could survive in the chicken gut under various pH and anaerobic conditions. After assessing all of the results, two microorganisms were chosen as final probiotics and evaluated in-vivo to ensure that they are safe for chickens and have a positive effect on their health

1.5 Thesis organization

There are five chapters in this dissertation; a concise initial chapter (Chapter 1) outlines the research background, research objective, novelty, research problem, and thesis organisation.

The essential properties of each area of this research, as well as a brief introduction to each, are covered in Chapter 2.

This work's research methodology is described in Chapter 3. From producing sample stock solutions to the final phase of proximity analysis, this chapter discusses every step and procedure of the research.

In Chapter 4, all of the findings and discussions from the entire research study are presented in detail.

Overall, Chapter 5 summarises all of the findings from this study. At the end of this chapter, there are also recommendations for future research.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The poultry industry, commercial poultry feed, antibiotic growth promoters, and the use of probiotics in the poultry business will all be covered in this chapter. In addition, the in-vitro and in-vivo investigations are thoroughly elaborated in this chapter. Finally, a remark will be placed.

2.2 Poultry agriculture and industry

Poultry farming is a method of raising tamed birds such as chickens, ducks, turkeys, and geese for the purpose of producing meat or eggs for human consumption. Malaysia wants to export at least 30% of broiler chickens by 2030, the poultry (chicken) industry in Malaysia is partitioned into two key productions, broiler, and eggs [19]. In Malaysia, the poultry industry is the most important livestock subsector, a few of the main reasons for the rapid spread of chicken farming in Malaysia is that it provides a low-cost source of protein that is socially acceptable to Malaysia's multi-ethnic population [20]. Malaysia's capacity to produce chicken increased to 106% for poultry meat and 115% of the demand for chicken eggs [21] [22]. There are an average of 2606 broiler grower farms producing 767 million poultry per year [23]. The total poultry presence in the world (chicken, duck, goose, guineafowl, and turkey) was approximated to be around 27.9 billion heads in 2019, as per data from the United Nations Food and Agriculture Organization (FAO) [24]. Chickens account for most of this presence, making for approximately 93 percent [24]. The expense and internationalisation of chicken and its products will be affected by strong production

competition and cost variations from around the world [25]. The poultry industry's current top priorities have already been disease control, high output, quality of products, and fair manufacturing costs [25].

2.3 Gastrointestinal tract mechanism and gut Microbiota of chicken

Chickens' GI (Gastrointestinal) tracts are home to a rich and complex microbiome that aids in digesting and nutrition absorption, as well as immune system development and pathogen elimination [26]. The crop, proventriculus, gizzard, duodenum, jejunum, ileum, caeca, large intestine, and cloaca are all parts of the chicken's GI tract [27]. The pH values in the chicken intestine fluctuate from crop to cloaca, as shown in figure 1.1. Bacteria dominate the gastrointestinal compartments of chickens, which are densely populated with microbial community (Bacteria, fungi, Archaea, protozoa, and virus) [28]. The intestinal microbiota can build a physical shield by adhering to the mucosal membranes of enterocytes, reducing the chance of harmful bacteria colonisation[28], [29]. The host benefits and pays for a healthy gut bacteria community [30], [31]. The microbiota generates nutrients and energy such as vitamin, amino acids, and short chain fatty acids (SCFA) from the unprocessed diet in the distal gut (i.e., ceca and colon), which finally get supplied to the host [30], [31]. SCFAs provide energy to the animals and can increase gut epithelium cellular proliferation, boosting the absorption surface of the gastrointestinal tract [30]. Microbes battle with the host seeking energy and protein in the proximal gut (gizzard and small intestine). Microbes create toxic compounds and catabolize bile acids in both the proximal and distal guts, which may inhibit growth and reduce fat digestion in the birds, respectively [31]. Dysbiosis is a term used to describe an unbalanced gut microbiome. Dysbiosis is described as a qualitative or quantitative mismatch of gut microbiome in

the small intestine, which can result to a chain of events in the GIT, such as decreased intestinal barrier (e.g., thinning of the intestinal wall) and poor nutrient digestibility, raising the risk of bacterial proliferation and inflammatory responses [32].

2.4 Antibiotic growth promoter in poultry industry

Antibiotic growth promoters are extensively used in poultry industry to increase weight gain [33]. A significant number of antibiotics used in the poultry industry are also used in human medicine [34]. Antibiotics are administered to broiler chickens through their food during their growing stage to prevent diseases and increase broiler growth [35]. Antibiotics are commonly used to treat individuals or small groups of animals with heavier doses of antibiotics for a short period of time, whereas prophylactic use can involve treating large groups of animals with moderate doses of antibiotics for extended periods of time [36]. Sub-therapeutic dosages of some known antibiotics or substances have poorly characterised antibacterial activity are used as growth promoters for very long periods of time, possibly for even the entire existence of the animals [36] Antibiotic resistance occurs as a consequence of microbial population being exposed to such drugs. Antibiotic overuse and abuse have pushed the resistance problem in human medicine, but it appears that preventive usage, and especially growth promoter use, have contributed most to the formation of resistant bacteria in animals[36]. Issues about the adverse effects on the environment and public health associated with the emergence of antibiotic resistance in highly contagious bacterial pathogens as a result of therapeutic and/or non-therapeutic antibiotic use have urged a global boost for more strictly enforced antibiotic use in food animal production [33].

2.5 Probiotics and their use in the poultry industry

2.5.1 Probiotics for poultry

Probiotics are living microorganisms that are advantageous to health, particularly in the digestive tract. Organisms are typically referred to as "good" or "friendly" microorganisms since they aid in gut health [37]. Probiotics promote broiler chickens' development and production of more eggs, protect them from diseases, boost their immune systems, strengthen their bones, and combat parasites [38]. Probiotics have a big influence on the gut microbiome's diversity and activity. Probiotics' hypothesised methods for achieving these effects include competing for resources with other microorganisms, binding sites, and receptors on the intestinal mucosa, and suppressing the development of other germs by releasing antimicrobial compounds [39]–[41]. Antibiotics and probiotics deliver antibacterial compounds at similar levels of efficiency as bacteriocin, organic acids, short-chain fatty acids (SCFA), or hydrogen peroxide (with intestinal pH alteration and in conjunction with glucose) [42].

2.5.2 Probiotics in poultry industries

When compared to broiler chicks fed with regular feed, studies show that broiler chicks treated with probiotics have a considerable increase in body weight, both vaccinated and non-vaccinated [43]. Supplementing chickens with probiotics enhanced weight gain, feed conversion ratio, and egg production while lowering mortality and disease rates [44], [45]. Probiotics, which operate as antibiotic replacements in the poultry farming, have grown in popularity in the realm of nutritional supplements and feed additives in recent years, also with the introduction

of probiotics, the phrase "immunity originates from the gut" has taken on new meaning in the poultry industry [46].

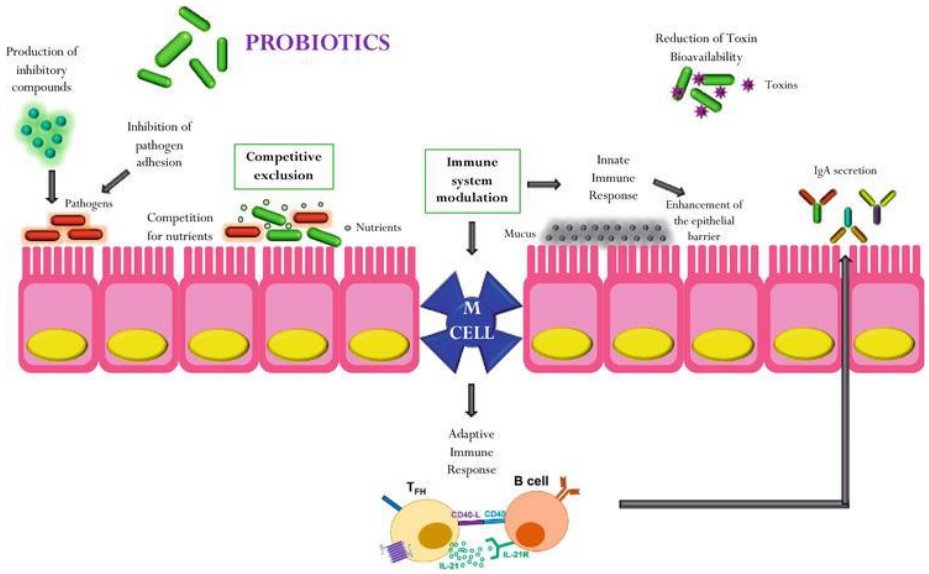

Figure 2.1: Probiotics' mechanisms for immune system modulation of poultry [47]

Probiotics can prevent pathogens through two main mechanisms [48]–[50]: competitive exclusion and modification of the host immune system (fig 2.1). Mechanisms of competitive exclusion include the synthesis of inhibitory substances such as hydrogen peroxide, bacteriocins, and defensins (Pan & Yu 2014; Tiwari, Tiwari, Pandey, & Pandey 2012); adherence of pathogens is inhibited (Tiwari, Tiwari, Pandey, & Pandey 2012); Nutritional competition (Kabir et al. 2004); Bioavailability of the toxins is reduced [49]. Both innate and adaptive immune responses are involved in the regulation of the host immune system [48]. B and T cells are responsible for inducing an antigen-specific action and producing antibodies in the adaptive immune response [48], [53] Physiochemical defences (innate immunity), which including intestinal epithelial cells (IEC), on the other hand, constitute the first line of defence against pathogens and diseases. Probiotics, which stimulate the

production of mucus and antimicrobial peptides like defensins, can enhance the function of the intestinal barrier by enhancing the formation of mucus and antimicrobial molecules like defensins [54], [55].

A variety of probiotic products are currently offered on a global scale. They are single strain, multi-strain, multi-species, and symbiotic products, and they come under many categories [56]. There are also a few multi-genus goods on the market, but they are uncommon due to the difficulty of cultivation and stabilisation. Sporulated Bacillus spp. and Lactic acid generating bacteria are the two main groups into which most commercial probiotic products fall [56].

2.6 Fermented foods and microbes

Fermented foods are described as foodstuffs or drinks developed by the controlled growth of microorganisms and the enzymatic conversion of dietary components [57]. Fermented foods include potential beneficial microbes such as lactic acid bacteria [57]. Fermentation has long been used to preserve and improve food's shelf life, taste, textures, and functional characteristics [58]. Fermented foods have long been known for their nutritional benefit, and there is some solid evidence that fermentation boosts the nutrient quality of foods [58]. Fermented foods provide a variety of functions and advantages that cannot be easily found in other diets. Fermented food products possess anti-fungal, antioxidant, anti-microbial, anti-diabetic, anti-inflammatory, and anti-atherosclerotic properties, among others [59]. There are lots of fermented foods, such as yoghurt, kefir, tempeh, kimchi, natto, belacan, etc.

2.6.1 Fermented soya cake (Tempeh)

Fermented soya cake is also known as tempeh, a Southeast Asian food. Tempeh contains no cholesterol and is an excellent source of B vitamins, iron, calcium, fibre,

and other minerals [60]. Tempeh is a complete protein source, that is, it contains all nine of the necessary amino acids required by human body for strong bones and muscles, it also has health promoting phytochemicals like isoflavones and saponins [60].

To begin with fermented soya cake production, soak the soya beans for at least 8 hours in a sufficient amount of water. The hulls of the soaked soya beans rise to the surface of the water when gently stirred. Water must be drained to release the hulls of the soya beans. Water must be repeatedly poured and drained until most of the hulls have been removed. The soya beans must next be cooked for 30 minutes in a large pot with lots of water and vinegar. The water must then be drained, and the cooked soya beans must be thoroughly dried by patting them dry with kitchen paper and leaving them to dry entirely. Allow the soya beans to cool to below 35 degrees Celsius before using. After that, 1 tsp tempeh starter (rhizopus moulds) should be sprinkled over the soybeans, and the cooked beans should be thoroughly mixed. Using a broad, thick needle, punch 1-inch-apart holes in plastic zip-lock bags. The perforations allow the mould to breathe, thus this is an important phase. Finally, the plastic packages must be kept in a warm place for 36–48 hours, at around 30 degrees Celsius. The plastic packets should be entirely filled with white mycelium after 36–48 hours, firming up the soya beans into one piece, similar to a cake, that may be withdrawn in whole portions [61].

2.6.2 Fermented shrimp paste (Belacan)

Fermented shrimp paste is known as belacan, it is also one kind of Southeast Asian food. Because belacan contains microorganisms that produce vitamin K2, it reduces

dental erosion and strengthens enamel, it also produces Mk-7, a natto-like substance [62]. Mk-7 is a vitamin k homologous [63].

Firstly, fresh and small shrimps need to be chosen. Then the shrimps need to be washed and cleaned with salt water. After that, the shrimps need to be mixed with salt. The right amount of salt will make the Belacan last longer and, most importantly, will not produce a bad smell. Then the mixture must be placed in a container or bucket (depending on the quantity) overnight, and then need to wrap it with a special transparent plastic wrapper. On the next day, the contents need to be taken out and dried under the sun. This drying process would take about 3 days, depending on the weather. Containers need to be kept in the fridge at the end of each day to continue the drying process the next day. Once it is semi-dried out, the dried shrimps are then blended or pounded into a paste before continuing to be dried under the sun for a day or two.

Table 2.1: Probiotics strain found in fermented food samples

Fermented food	Probiotic strain
Fermented soya cake (Tempeh)	*Lb. fermentum, Lb. plantarum, P. pentosaceus, W. confusa, Lb. delbrueckii ssp. delbrueckii, Lb. plantarum* [64], [65]
Fermented shrimp paste (belacan)	*B, pumilus, B. megaterium, B. licheniformis, B. firmus, B. alvei and B, mycodes.* [66]

2.7 Microbial culture, isolation, and morphology

2.7.1 Microbial culture

A microbial culture, also known as a microbiological culture, is a technique for increasing microorganisms by allowing them to grow in a specified growth media under carefully regulated laboratory conditions [67]. Microbial growth media, often known as growth media, is the medium used in microbial culture for microbial growth. A culture medium is a solid, liquid, or semi-solid that is used to support the growth of a population of microorganisms or cells through the process of cell proliferation [68].

Based on consistency in cultural media, there are three types. Solid medium comprises agar at a level of 1.5-2.0 percent or another mainly inert solidifying agent. Solid media has a physical framework that permits bacteria to grow in physiologically informational or beneficial ways, as colonies or in streaks, solid medium is helpful for isolating microorganisms or identifying the colony properties of the strain [69].

Agar with a concentration of 0.5 percent or less is used to make semisolid medium. Semisolid media has a smooth custard-like texture and can be used for microaerophilic bacteria culture or bacterial mobility testing [69].

These media contain specified levels of nutrients but no gelatine or agar to act as a gelling agent. Broth medium is used for a variety of applications, including the multiplication of a huge range of microorganisms, fermentation investigations, and other experiments [69].

General-Purpose Media, Enriched Media, Selective and Enrichment Media, Differential/ Indicator Medium, Transport media, Anaerobic media, and Assay media

are the classifications of bacterial culture media based on their functional usage or application [69].

2.7.2 Isolation

The separation of a variant from a spontaneous, diverse population of living microbes is referred to as isolation in microbiology[70].

2.7.3 Microbial morphology

The size, structure, and configuration of cells is referred to as morphology [71]. Examination of microbial cells necessitates not only the use of microscopes, but also the pre-treatment of the cells in a way that is acceptable for the type of microscope being used [71]. The typical magnification factor of a light microscope is 1000, with a maximum usable magnification of around 2000. Specimens can be seen in live, unstained preparations as a "wet mount" or after they have been stained using one of various procedures to emphasise particular morphological traits [71].

2.8 In vitro study and chicken gut

In vitro (meaning "in glass" or "in the glass") experiments are conducted with microbes, cells, or biomolecules outside of their basic biological setting [72]. Studies separating distinct sections of an organism from their normal biological settings allow for more detailed or convenient study than can be performed with whole organisms [72]. Organisms are very complex structural systems are composed of thousands or even millions of genes, biological macromolecules, RNA molecules, tiny chemical substances, inorganic materials, and compounds in an environment spatially organised by membranes and, in the context of multicellular animals, organ systems [73].

Because of its complexity, it is difficult to discover interconnections between individual elements and to investigate their underlying biological roles. In vitro work streamlines the system under investigation, allowing the investigator to concentrate on a limited number of features [74], [75]. For this study's in vitro analysis, a 37°C temperature, anaerobic condition, and seven distinct pH levels were considered and maintained as the in vitro analysis environment. The pH level of the chicken gut varies from pH 2.5 to pH 8 [76].

2.9 Gas chromatography- mass spectrometry (GCMS)

Gas chromatography–mass spectrometry (GC-MS) is an analytical method for identifying different substances within a test sample that combines the features of gas chromatography and mass spectrometry[77]. It can analyse and detect even minute amounts of a substance [78].

2.10 In vivo study and live chicken

In microbiology, the term "in vivo" refers to research conducted in a living organism rather than isolated cells [79]. The examination of the effects of particular chemicals in vivo is more accurate in a complex model, according to in vivo results [80]. It is simple to see all of a substance's benefits and side effects throughout all parts of the body [80]. It's a simpler method since there are fewer variables to regulate. In addition, it is more therapeutically relevant [80].

The experimental animal in this research were chickens. Chickens were monitored with caution and care throughout this sophisticated in vivo monitoring process. Their hygiene was carefully checked to ensure that they did not contract any infections and

could thrive in a healthy environment. Their experimental probiotic samples and feed water were delivered on schedule.

2.11 Protein analysis

Proteins are complex biomolecules or macromolecules that are made up of one or more long amino acid chains [81]. Proteins play a variety of roles in individuals, including stimulating metabolic events, DNA replication, reacting to stimuli, giving cells and organisms shape, and moving materials from one place to another [81].

Protein concentrations can be analysed using a variety of techniques. One of these is Kjeldahl method. Johann Kjeldahl, a brewer, invented the Kjeldahl technique in 1883 [82]. The Kjeldahl technique can be split into three simple steps: digestion, neutralisation, and titration [83].

2.12 Lipid analysis

A lipid is a macro biomolecule that may be dissolved in nonpolar solvents in biology and biochemistry [84]. Lipids have several activities, including energy storage, signalling, and functioning as building elements of cell membranes [85]. Cholesterol, triglycerides, and phospholipids make up the majority of the fat in diet. Some dietary fat is required for fat-soluble vitamins (A, D, E, and K) and carotenoids to be absorbed [86].

A variety of methods can be used to evaluate lipid or crude fat concentrations. The Soxhlet extraction technique is one of them.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

Beneficial microorganisms were isolated from fermented soya cake and shrimp paste. To ensure that the research was free of contamination, the entire procedure was carried out inside a laminar flow cabinet. The initial stage in this research is to prepare two stock solutions from fermented shrimp paste and fermented soya cake. For bacteria and yeast, two types of culture media were prepared. Except for the samples, all the equipment, distilled water, and prepared media were autoclave sterilized.

In this study, full chicken GIT conditions were meticulously maintained while researching in vitro investigation, demonstrating that selected several microbes may survive just fine throughout the condition like chicken's GIT. OD for the nineteen microorganisms (ten bacteria and nine yeast) were measured every hour for 24 hours during in vitro analysis. Following that, biomass testing was done. Throughout the procedure, liquid culture media was used. Later, the growth medium of the eight organisms that grew the fastest (three bacteria and five yeast) was filtered, and for extracellular media, GC-MS was performed to verify the chemical compounds produced. The toxicity of the chemical component, the kinetics of growth, survivability, and the best biomass of microbes were then compared, and only two microorganisms (one bacteria and one yeast) were chosen to produce probiotics for chickens, followed by an in vivo study and some more analysis of the chickens to ensure the probiotics' effect on their health.

3.2 Flow chart

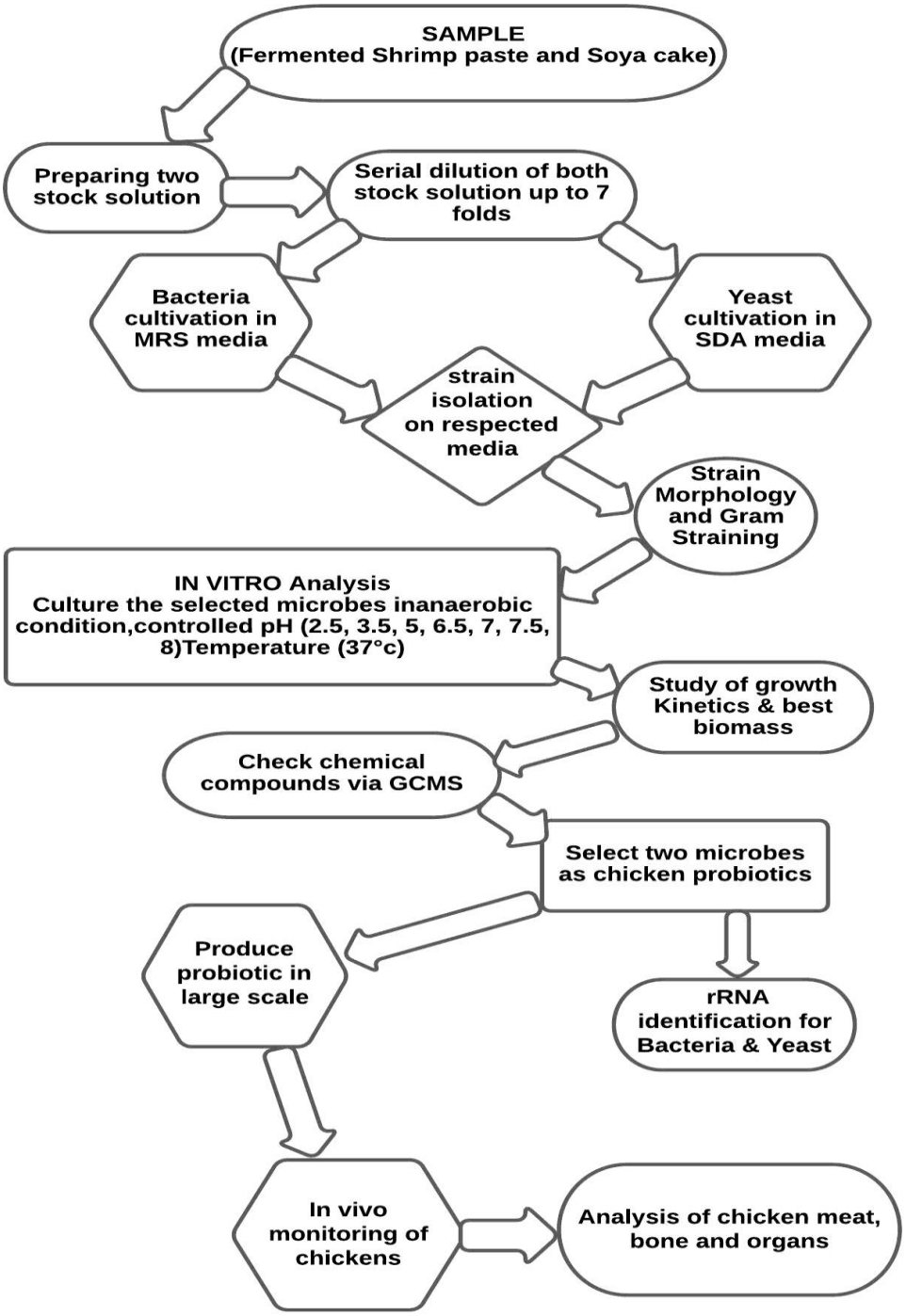

Figure 3.1: Flow chart

The whole methodology of this research project is depicted in figure 3.1.

3.3 Samples, stock solution preparation and serial dilution

3.3.1: Samples

Fermented shrimp paste and fermented soya cake were chosen as the study's samples. They were bought at a local supermarket.

(A) (B)

Figure 3.2: (A) Tempeh (B) Belacan

Photos of samples (A) Tempeh and (B) Belacan are shown in figure 3.2.

3.3.2 Stock solution preparation

In the first step, stock solution preparation was done with the fermented food samples. By diluting 1 gm of each sample with 10 ml of sterile distilled water separately two distinct stock solutions of soy cake and shrimp paste were produced. Sterile glassware and a sterile spatula were used. The whole stock solution preparation was done inside a laminar flow cabinet.

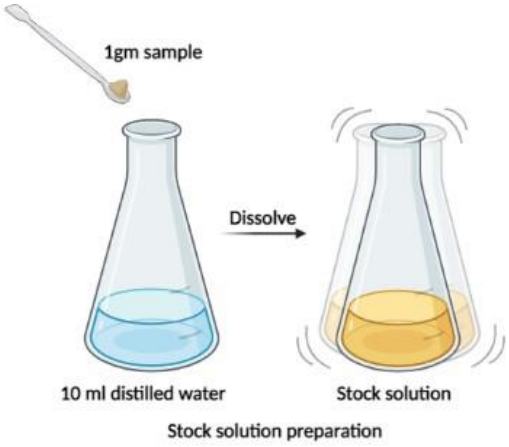

Figure 3.3: stock solution preparation

Figure 3.3 demonstrates the process of stock solution preparation.

3.3.3 Serial dilution

After making the stock solutions, both stock solutions were serially diluted seven times ten folds (10^7). That is, one millilitre of sample was diluted with nine millilitres of distilled sterilised water, and the process was repeated seven times (10^1, 10^2, 10^3, 10^4, 10^5, 106, and 10^7).

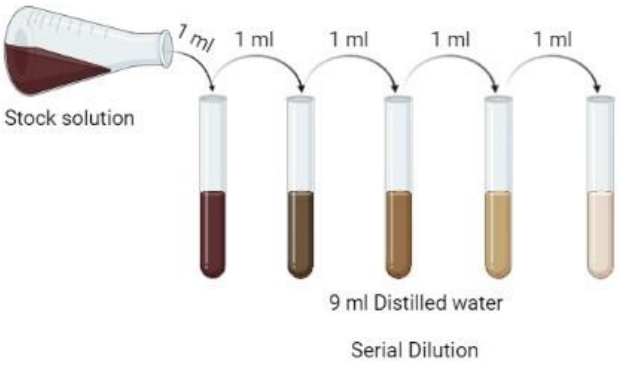

Figure 3.4: serial dilution

Serial dilution is frequently used to reduce the number of cells in a culture in order to make the process simpler to isolate single colonies for further research [87]. The

repeated dilution of two main samples led to the founding of 16 different samples. The process of serial dilution is shown in figure 3.4.

3.4 Culture and Isolation

Both the fermented soya cake and the fermented shrimp paste samples were subjected to bacterial and yeast cultures. For bacterial cultivation, MRS bacterial culture media was used, and SDA culture media was used for yeast cultivation. Both culture mediums were solid culture mediums.

3.4.1 MRS Media

MRS agar media is an abbreviation for De Man, Rogosa, and Sharpe agar media. This media is selective for bacteria. MRS agar was created with the goal of providing a defined medium that could be used instead of tomato juice agar for the culture of lactobacilli from a variety of sources [88]. This medium provides bacteria with a consistent amount of nutrients, amino acids, and vitamins. MRS agar is typically made up of components such as proteose peptone 10 gm/L, beef extract 10 gm/L, yeast extract 5 gm/L, dextrose 20 gm/L, polysorbate80 1 gm/L, ammonium citrate 2 gm/L, sodium acetate 5 gm/L, magnesium sulphate 0.1 gm/L, manganese sulphate 0.05 gm/L, dipotassium phosphate 2 gm/L, agar 10 gm/L and pH 6.5±2 [88].

3.4.2 SDA Media

Sabouraud agar, often known as Sabouraud dextrose agar (SDA), is a peptone-containing agar growth media [89]. Sabouraud's Dextrose Agar contains animal tissue digests (peptones), which provide a nutritive source of amino acids and nitrogenous compounds for the growth of fungi and yeasts, as an energy and carbon source, dextrose is added [90]. The pH is adjusted to around 5.6 to promote the growth of

fungi, particularly dermatophytes, and to slightly inhibit bacterial growth in clinical specimens. Fungal growth is aided by a high dextrose concentration and a low pH, which suppresses contaminating bacteria in test samples [90]. Dr. Raymond Jacques Adrien Sabouraud, a French physician, invented the Sabouraud Dextrose Agar (SDA) or Sabouraud agar medium. SDA agar is typically made up of dextrose 40 gm/L, peptone 10 gm/L, agar 20 gm/L and pH approximately 5.6 [90]

The ingredients for each media were dissolved in one litter of distilled water, then autoclaved before being poured into petri dishes. Autoclaving is essential for media sterilization when the intention is a contamination-free research output. The autoclave treatment takes 15 minutes at 121°C and 15 pounds of pressure. Following autoclaving, both media were poured into individual petri dishes, which were then placed inside a laminar flow cabinet and exposed to UV radiation to kill any contaminants. Following the solidification of the media, both yeast and bacteria were cultured on their respective selective media. The pour plate method was used to cultivate yeast and bacteria. Each diluted sample was put in a separate petri dish. The culture plates were then sealed with parafilm and incubated for 24 to 72 hours at 35°C for MRS plates (bacteria) and 30°C for SDA plates (yeast) to allow the microorganisms to grow. After nearly 60 hours, the petri dishes were removed from the incubator and small colonies of newly grown microorganisms were isolated.