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Methods for preservation and transcriptomic analysis of Lactobacillus Plantarum P1, P2 phage

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Abstract - The survival of Lactobacillus plantarum virulent phages P1 and P2 in three cryoprotectants: glycerol(15%), chloroform(20%), and dimethyl sulfoxide(7%) under different temperatures(4°C, -20°C, -80°C) were evaluated every half month. The study was undertaken to establish suitable preservation methods that could be used to study phage biological properties and genomic characteristics further. Transcriptomic properties of phage activity in different stages of lysis were also analyzed. This information may be used further to explore the relationship between bacteriophage and host bacteria. The results of this study showed that Lactobacillus plantarum virulent phage P1, stored for 60 days in all cryoprotectives at all temperatures, exhibited a survival titer greater than 10^7 PFU/mL (initial titer 10^8 PFU/mL). The optimum cryoprotectant used was glycerol at -80°C. In this respect, the phage could be preserved for 10 months with a 10^5 PFU/mL titer. Maintenance of Lactobacillus plantarum virulent phage P2 under different storage conditions was evaluated. Preservation of the phage for up to three months was achieved at 4°C using all cryoprotectants. In this respect, the phage titer was greater than 10^8 PFU/mL. For long-term phage maintenance (10 months), the optimum

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preservation conditions at -80°C included 15% glycerol or 7% dimethyl sulfoxide as cryoprotectants. Under these conditions, the phage titer was greater than 10^7 PFU/mL. The infective phases of phage P2 and its host strain were investigated using transcriptomic analyses. It was observed that when phage P2 infects its host strain, it can control the replication and assembly process of phage by regulating key enzymes (such as prolong factor EF-Tu, EF-G, EF-Ts; translation starting factors IF1, IF2, IF3; RNA synthesis enzyme rpoA, rpoB, rpoC; signaling recognizing particles Fts Y, Ffh; as well as releasing factor RF1).
Keywords - L. plantarum IMAU10120, bacteriophage, phage preservation, transcriptomic Analysis

1. INTRODUCTION

In recent years, with the rapid development of genomics, it has been found that phage is a component of bacteria; it co-evolved with bacteria and can improve the adaptability of bacteria. At the same time, phage also has many practical applications, such as the use of high-density phage to select phage-resistant mutant strains to replace the original strains in industrial production, which fundamentally solves the problem of fermentation failure caused by phage infection in the fermentation process of Lactobacillus plantarum. Bacteriophages can also be used as a carrier to deliver nucleic acid fragments, and they are widely used in the gene excision of Lactobacillus plantarum. Bacteriophages are highly specific and can be used as therapeutic agents instead of antibiotics to solve the problem of bacterial resistance [1]. Bacteriophages used in aquaculture can improve the survival rate and growth rate of animals such as livestock, poultry, and fish; in medical treatment, they can reduce the morbidity and mortality of patients with bacterial diseases; and in the food industry, they can extend the freshness period of food [2]. Therefore, bacteriophages have broad application prospects. However, the bacteriophage is a microscopic organism, is a strictly obligate parasite, and cannot reproduce and grow independently after leaving the host bacteria; its preservation and activity will be affected by a variety of different factors, such as temperature, protective agents, etc., so from its preparation to the actual application of the period of storage conditions are crucial. To maximize its effect, people have tried a variety of different preservation conditions preservation methods. The diversity of bacteriophages and their sensitivity to various storage conditions and storage media content vary, so there is no one-size-fits-all storage method.

The transcriptome is the collection of all RNA transcribed by a particular tissue or cell at a specific developmental stage or functional state, including mRNA and non-coding RNA [3]. As one of the new omics techniques, transcriptomics studies reflect the genes expressed under different physiological states, tissue types, and environmental conditions [4]. Therefore, transcriptome analysis has become a powerful tool for studying biological stress's physiological mechanism [5]. The transcriptomic study during bacteriophage infection can initially explore the possible mechanism of bacteriophage infection in host bacteria, which is of great significance for studying the relationship between bacteriophage and host bacteria. In this paper, the virulent phage P1 and P2 of Lactobacillus plantarum, isolated and purified from abnormal fermentation solution, were preserved with different protective agents under different

temperature conditions. The optimal preservation method was explored by calculating and comparing the titers of phages before and after different preservation methods. This will lay a research foundation for further determination of its biological characteristics and evaluating its application value in the future. At the same time, transcriptomic studies on different periods of phage infection can reveal the characteristics and modes of phage infection to host bacteria and further explore the relationship between phage and host bacteria. To provide a theoretical basis and data support for phage control and screening of phage-resistant strains in the dairy industry. At the same time, it is essential to use phages in further research by conducting transcriptomic studies at different stages of infection to control the harmful effects of bacteriophages in dairy industries, to determine their biological properties, and to provide theoretical basis and data support for the detection of anti-phage strains.

2. THEORETICAL BACKGROUND

2.1 LACTOBACILLUS PLANTARUM IMAU10120

Lactobacillus plantarum is a bacterium of the genus Lactobacillus of the Lactobacillus family. It is straight or curved rod-shaped, has few flagellates, and can exercise. It belongs to Gram-positive bacteria and has no spore formation. It is widely distributed in animal and plant feed, manure, milk, and dairy products [6]. Its main feature is that it can ferment lactose to produce lactic acid. Lactobacillus is used commercially primarily in making yogurt and cheese, but also in product-fermented vegetables (pickles and pickles) [7], beverages (wine and juice), sourdough bread, and some sausages [6]. The optimal growth temperature is 30℃~35℃, and the optimal pH is 6.5 [8-9]. It is commonly found in human digestive tracts. Lactobacillus plantarum helps to improve immunity, maintain intestinal flora balance, reduce serum cholesterol content [10], prevent cardiovascular disease, alleviate lactose intolerance, and inhibit the formation of tumor cells, as well as anti-oxidation, anti-intestinal aging, and other effects [11].

Lactobacillus plantarum IMAU10120 was isolated from traditional fermented yogurt samples collected by grassland herders in Urat Zhongqi, Bayannur City, Inner Mongolia, China. This is a probiotic strain with excellent tolerance to stomach acid, intestinal fluid, and bile salts [12]. The genome consists of 3.03 Mb circular chromosomes and seven plasmids, named LBPp1 to LBPp7 [13].

Bao, Zhang, Wang, et al. conducted an in-depth study and systematic evaluation of its probiotic function and the mechanism by using genomic means, in vitro tests, and human tests, respectively. The results showed that Lactobacillus plantarum IMAU10120 could significantly inhibit the growth of intestinal pathogens and had good tolerance to gastrointestinal digestive fluid and bile salts [12-14]. In addition to high acid and bile resistance, it has many other probiotic properties, Such as good aggregation, antibacterial activity, and storage stability [15]. This strain can also improve human gastrointestinal health by regulating the host intestinal microbiota and the secretion of total bile acids and short-chain fatty acids [16]. Lactobacillus plantarum IMAU10120 can reduce the levels of serum total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol, inhibit the increase of high-density lipoprotein cholesterol levels, and play a role in lowering blood lipids. In addition, Lactobacillus plantarum

IMAU10120 also plays a beneficial role in high-fat diet-induced oxidative stress, reducing liver fat accumulation and protecting healthy liver function [17]. These results suggest that Lactobacillus plantarum IMAU10120 may be a potential therapeutic agent for the control of hyperlipidemia [18].

2.2 BACTERIOPHAGE

A bacteriophage is a virus that infects a bacterial cell. There are many types of phages worldwide, and they can be classified as beneficial and virulent. Lactobacillus bacteriophages are the leading cause of lactic acid bacteria fermentation failure, and this phage is a frequent problem in the dairy industry. After infecting the lactic acid bacteria, the bacteriophage multiplies inside the bacterial cell and destroys the lactic acid bacteria. Because of this, the fermentation of milk is slowed down, or fermentation does not occur, causing substantial economic losses to the dairy industry. Bacteriophages are tiny organisms that cannot reproduce and grow independently after breaking away from the host bacterial cell. Therefore, temperature and preservatives have an essential effect on the storage of dairy products.

In 2015, a strong bacteriophage P1 was isolated from the abnormal fermentation broth of L. plantarum IMAU10120. The bacteriophage has a head diameter of 71.7 ± 3.0 nm, a tail length of 272.0 ± 3.0 nm, and a width of 11.3 ± 1.5 nm. It is a long-tailed bacteriophage with a latency period of 45 minutes, a lysis period of 45-90 minutes, and an average cleavage amount of 132.88 ± 2.37 PFU [19-20]. External environmental conditions such as temperature, pH value, and concentration of divalent cations all affect the survival rate and adsorption capacity of bacteriophage P1. The optimal adsorption temperature for bacteriophage P1 is 30 ℃~42 ℃, and the optimal adsorption pH is 6-8. Calcium ions can promote adsorption, while magnesium ions inhibit adsorption in host bacteria [21]. Phage P1 is sensitive to high temperatures and can be completely inactivated after being treated at 90 ℃ for 5 minutes. It has varying degrees of tolerance to commercial sterilization agents, and after 60 minutes of treatment, high concentrations of alcohol substances and peracetic acid cannot completely inactivate it; Research has shown that bacteriophage P1 is more sensitive to sodium hypochlorite, and treatment with 800 mg/L sodium hypochlorite for 60 minutes can lead to its complete inactivation [22]. In 2017, a virulent bacteriophage P2 was isolated from an abnormal fermentation broth of the same strain of phage P1, which had a low photometric content of a long-tailed bacterium, and the fermentation broth was a clear phylum B1 type, 216.67±3.00 nm in length, and a non-contractile tail. It is 12.25±3.00 nm wide, 66.67±3.00 nm head diameter icosahedral symmetric structure 30 minutes, degradation time 30~135 minutes, average degradation amount 214.49±3.98 PFU. At pH 8, the viability of this phage is the highest, and the optimal growth temperature is 37~42°C, and Mg2+ inhibits absorption. In addition, although chloramphenicol affects the synthesis of cell membrane recognition proteins, it does not affect the absorption capacity of bacteriophage P2. Still, the absorption capacity is more significant than 90.19% for 30 minutes. P2 bacteriophage is temperature-tolerant and is inactivated by treatment at 90°C for 10 minutes or 72°C for 40 minutes. This bacteriophage is not sensitive to peracetic acid but is sensitive to isopropyl alcohol and sodium hypochlorite and is wholly inactivated by 800 ppm sodium hypochlorite for 30 min [23].

2.3 MECHANISM OF PHAGE INFECTION

Like other viruses, bacteriophages need to infect a host cell. This process is known as the lysogenic lifecycle. The life cycle steps include attachment, cytoplasmic entry, protein synthesis, DNA copying, and phage assembly. It recombines with a specific area of the bacterial chromosome during the lysogenic cycle. The prophage initiates a lytic cycle on its initiative, or in response to particular physical or chemical stimuli, degrades and eliminates the host bacteria, and multiplies. To self-reproduce and lyse host bacteria directly, virulent bacteriophages copy and replicate their genes [24]. Shen Yanjie et al., studied the lysis of host bacteria by mycobacterial phage and discovered the lysogenic and lytic cycles of lysogenic phage. Their study showed that binding of mycobacterial lysogenic phages to host bacteria requires phage genome (attP), host bacterial genome attachment site (attB), integrase (Int), and integration host factor (mlHF). Some lysogenic phages undergo a lysis cycle; they assemble to reproduce new phages by copying and synthesizing phage genes. Then, the burst of bacterial cells due to the combined action of (Lysin), and Perforin (Holin), and phages capable of further reproduction are released [25]. In 2013, Tingting Guo discovered the infection mechanism of Lactobacillus phage in his research, in which perforin polymerizes on the host cell membrane to form a homotetramer and create a needle-shaped hole. Lysine is then converted from an inactive form to an active state to degrade cell wall peptidoglycan and lyse the host bacteria [26]. Due to the differences between phages and their host bacteria, each phage has its unique infection mechanism.

Transcriptomic studies examine how genes are affected by different physiological states, tissue types, and environmental conditions [27]. Therefore, transcriptome analysis has become essential for studying biological stress and physiological mechanisms [28]. Transcriptomic studies can study the mechanism of phage infection in host bacterial cells, which is necessary for studying the relationship between phage and host bacteria.

3. RESEARCH METHODOLOGY

3.1 DETERMINATION OF PHAGE TITER

The study was conducted at the Dairy Biotechnology and Engineering Laboratory of the School of Food Science, Inner Mongolia Agricultural University. Virulent phages P1 and P2 isolated from Lactobacillus plantarum IMAU10120 in 2015 and 2017 were used in the study. Phage P1 and P2 were stored in sterile 15% glycerol, 20% chloroform, and 7% dimethyl sulfoxide as preservatives at 4 ℃, -20 ℃, and -80 ℃ for 10 months. During this storage period, the bilayer plate method counted phages twice a month and recorded results.

Data from the study on bacteriophage storage were analyzed using one-way analysis of variance (ANOVA) in SPSS Statistics 20.0. Each experiment was performed in triplicate and analyzed for differences using Microsoft Office Excel 2016.

3.2 EXTRACTION OF TOTAL GENOMIC RNA FROM SAMPLES, CREATION OF SEQUENCING DATABASE

In this study, only host bacterial RNA degraded by infected phage was extracted after 0, 5, 15, 25, 80, and 120 min. Nanodrop 2000 was used to detect the concentration and purity of the extracted RNA, agarose gel electrophoresis was used to detect RNA integrity, and Agilent 2100 was used to determine RIN values, respectively. The total RNA required to generate one database is 1 μg, the concentration is ≥50 ng/μl, and the OD260/280 is between 1.8 and 2.2. Short sequence fragments are intended for sequencing using the Illumina HiSeq platform. Adding a fragmentation buffer can enable fragmentation of the mRNA into tiny pieces of approximately 300 bp. Subsequently, six-base random primers (random hexamers) are added under reverse transcriptase to synthesize single-stranded cDNA utilizing mRNA as a reverse template. The next step is to create a stable double-stranded structure through second-strand synthesis. The ends of the double-stranded cDNA construct are sticky; therefore, blunt ends are created by adding an end-repair mixture. To connect the Y-shaped adapter, finish the adapter ligation, and carry out library enrichment, an "A" base is attached to the 3' end. The target band was removed from a 2% agarose gel (Certified Low Range Ultra Agarose) after 15 cycles of PCR amplification. Once the quantity of TBS 380 (Picogreen) has been determined, mix it by the data ratio and place it on the machine. cBot was utilized for bridge PCR amplification, while Illumina Hiseq was employed for sequencing to produce clusters. To reduce read mistakes, every cDNA molecule in the database underwent paired-end sequencing.

3.3 QUALITY CONTROL AND SCREENING OF THE ORIGINAL SEQUENCE, SEQUENCE COMPARISON ANALYSIS

Raw transcriptome sequencing data performed using the Illumina Hiseq sequencing platform may contain sequencing adapter sequences, low-quality reads, etc., severely impacting subsequent data analysis. Therefore, at first, it is necessary to remove non-target sample sequences to ensure the accuracy of the following analysis. To do this, first, remove the adapter series in the reads and trim the bases with a quality value of less than 20 at the 3' end by using the software SeqPrep(https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle). Reads with an N ratio greater than 10% were discarded, and adapters and sequences less than 20 bp in length were removed after quality trimming. Reads obtained with the Illumina Hiseq sequencing platform are short and have low insertion and deletion errors. Bowtie2, the currently most authoritative short sequence comparison program, was chosen to complete this part of the analysis, which generally allows two base mismatches. This process uses the Bowtie2 (http://www.bowtie-bio.sourceforge.net/ bowtie2/manual) BWT (Burrows-Wheeler Transform) algorithm.

3.4 FUNCTIONAL ANNOTATION OF DIFFERENTIAL GENES

Genes can be categorized using the KEGG database based on the pathways they participate in or the functions they carry out. For the pairs of differentially expressed genes, the differential genes are shown on the KEGG pathway diagram to demonstrate the differential genes KEGG Annotate pathway diagrams, with one of the samples serving as a control.

3.5 DATA PROCESSING

The base identification analysis software Illumina Casava 1.8+ or bcl2fastq software converts the raw image data obtained from sequencing on a high-throughput sequencing platform (like the Illumina HiSeq sequencing platform) into base information. FastQC software is used for shutdown. The raw data is then filtered using NGSQC software to remove low-quality reads and reads tainted by sequencing adapters and HISAT software to align each sample's clean reads to the reference gene to control the data quality. Use Cuffdiff and edgeR software to analyze differentially expressed genes between samples; use online resources like Gene Ontology and the Kyoto Encyclopaedia of Genes and Genomes database to analyze differential genes; and use RSEM to process sequencing data to calculate the expression of genes or transcripts. Analyze the enrichment.

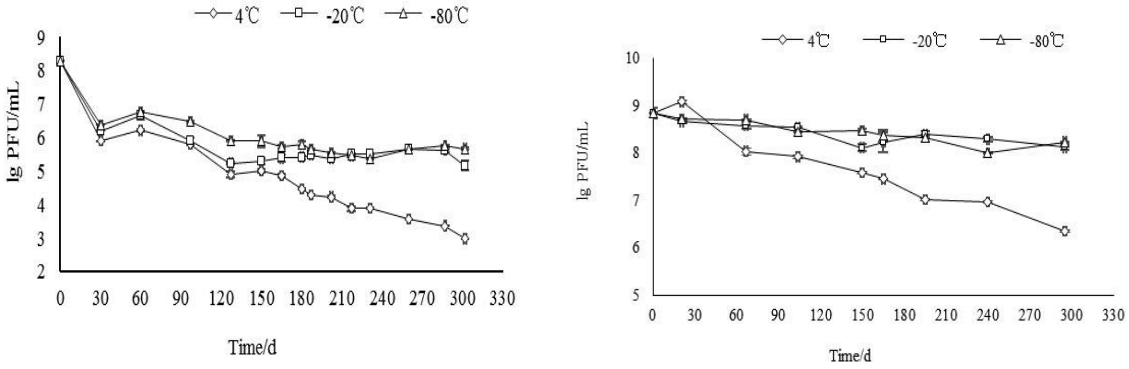
4. RESULTS AND ANALYSIS OF THE STUDY

4.1 PRESERVATION EFFECT OF TEMPERATURE AND DIFFERENT PROTECTIVE AGENTS ON LACTOBACILLUS BACTERIOPHAGE

4.1.1 P1 AND P2 PHAGES PRESERVED IN 15% GLYCEROL AT DIFFERENT TEMPERATURES

As shown in Figure 1, the initial titer of phage P1 was 1.94×108 PFU/mL. When stored at 4℃ and -20℃ with the final concentration of 15% glycerin as a protective agent for three months, the titer decreased by one logarithmic degree, while the basic preservation titer remained unchanged at -80℃. After four months of storage, the survival rate of bacteriophages stored at 4℃ and -20℃ was significantly reduced, and the titer of bacteriophages was reduced to 6.20×10^4 PFU/mL and 1.19×10^5 PFU/mL, respectively, while the titer of bacteriophages stored at -80℃ could maintain 10^6 PFU/mL. After ten months of storage, the titers of bacteriophages stored at 4℃, -20℃ and -80℃ were reduced to 9.6×10^2 PFU/mL, 1.44×10^5 PFU/mL and 4.70×10^5 pFU /mL, respectively. It can be seen from the figure that glycerin has a better preservation effect on phage P1 at -80℃ than at other temperatures. Therefore, when glycerin is used as the protective agent of phage P1, the optimal storage temperature is -80℃.
As shown in Figure 2, the initial titer of phage P2 at the initial preservation stage was 6.95×10^8 PFU/mL. With the extension of storage time, the titer of phage P2 under any conditions decreased. Using glycerin as a protective agent, the titer of phage P2 decreased by one order of magnitude after storage at -80℃ for eight months, and the titer of phage P2 was still 10^7 PFU/mL after storage for ten months, which was only reduced by one order of magnitude. After storage at -20℃ for six months, the titer of P2 decreased by one order of magnitude. After storage at -20℃ for ten months, the titer of P2 reached 10^7 PFU/mL but was lower than that at -80℃. After storage at 4℃ for four months, the titer of phage P2 decreased by one order of magnitude. After storage at ten months, the titer of phage P2 decreased by two orders of magnitude to 10^6 PFU/mL. The storage effect of glycerin on phage P2 at -80℃ was significantly better than that at the other two temperature conditions ($P\leq0.05$). In this paper, 15% glycerin was used as a protective agent to preserve phage P1 and P2 at different

temperatures. The results showed that for phage P1 and P2, -80℃ was the best storage temperature. This may be because glycerin can combine with water as a hydrogen bond to form non-freezing water, thus making the aqueous solution sticky. The freezing process is slow, and



the damage to the phage structure is reduced.

Fig. 1, 2 The phage P1 and P2 volume is stored in 15% glycerol at different temperatures.

4.1.2 PHAGE P1 AND P2 PRESERVED IN 20% CHLOROFORM AT DIFFERENT TEMPERATURES

As shown in Figure 3, when chloroform was used as a protective agent for phage P1 for three months, both 4℃ and -20℃ reduced its titer by two logarithmic levels, while -80℃ only reduced it by one logarithmic level. In the 4th month, the titer of phage P1 stored at 4℃ and -20℃ decreased significantly to 10^4 PFU/mL, while the titer of phage P1 stored at -80℃ could still reach 10^6 PFU/mL, and the preservation effect was significantly higher than that of the other two groups. After ten months of storage, the titer of phage P1 stored at 4℃ and -20℃ has been reduced by six logarithmic levels, and the survival rate is meager, while the titer of phage P1 stored at -80℃ can reach 10^5 PFU/mL, which has significant difference compared with the other two storage methods ($P \leq 0.05$). Therefore, for chloroform, the optimal temperature for the optimal preservation of phage P1 is -80℃.

As shown in Figure 4, when chloroform was used as a protective agent for phage P2, the survival rate of phage P2 gradually decreased with the extension of storage time. After three months, the titers of the bacteriophages stored under each storage condition began to decline significantly. The titer of phage P2 stored at -80℃ decreased by one order of magnitude after seven months and was still 10^7 PFU/mL after ten months, which was only reduced by one order of magnitude. The titer of phage P2 stored at -20℃ decreased by one order of magnitude after four months and was 10^7 PFU/mL after ten months, which was lower than that of phage P2 stored at -80℃. The titer of phage P2 stored at 4℃ decreased by one order of magnitude after four months and 10^6 PFU/mL after ten months, which decreased by two orders of magnitude. Within three months, there was no significant difference in the preservation effect of chloroform at 4℃, -20℃, and -80℃ ($P \leq 0.05$). After four months of storage, the storage effect of chloroform on phage P2 at -80℃ was significantly better than that of the other two groups. Therefore, the optimal temperature for preserving phage P2 by chloroform was -80℃. In this study, 20% chloroform was added to phage lysate and stored at 4℃, -20℃ and -80℃,

respectively. The results showed that the lower the temperature, the better the preservation effect of chloroform on phage P1 and P2. Among them, -80℃ was the most suitable temperature for preserving phages P1 and P2 by chloroform. As shown in Figure 3, when chloroform was used as a protective agent for phage P1 for three months, both 4℃ and -20℃ reduced its titer by two logarithmic levels, while -80℃ only reduced it by one logarithmic level. In the 4th month, the titer of phage P1 stored at 4℃ and -20℃ decreased significantly to 10^4 PFU/mL, while the titer of phage P1 stored at -80℃ could still reach 10^6 PFU/mL, and the preservation effect was significantly higher than that of the other two groups. After ten months of storage, the titer of phage P1 stored at 4℃ and -20℃ has been reduced by six logarithmic levels, and the survival rate is meager, while the titer of phage P1 stored at -80℃ can reach 10^5 PFU/mL, which has significant difference compared with the other two storage methods ($P \leq 0.05$). Therefore, for chloroform, the optimal temperature for the optimal preservation of phage P1 is -80°C.

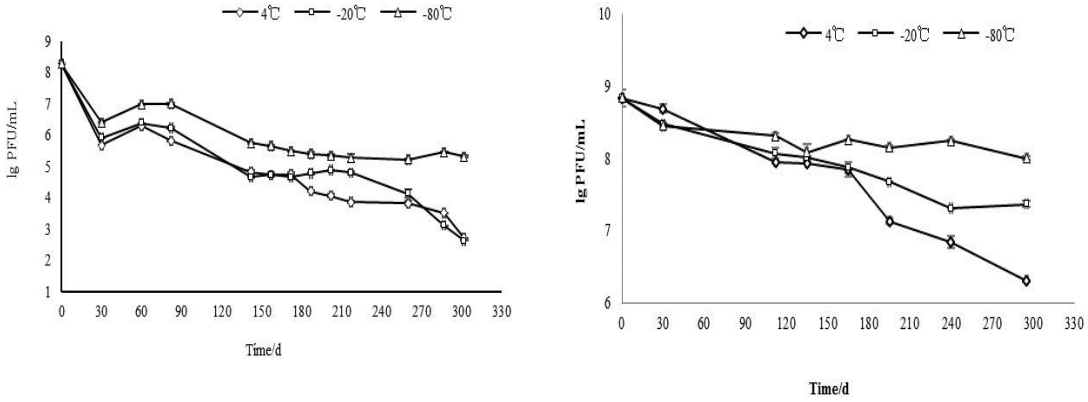


Fig. 3, 4 The phage P1 and P2 volume is stored in 20% chloroform at different temperatures.

4.1.3 P1 AND P2 PHAGES PRESERVED IN 7% DIMETHYL SULFOXIDE AT DIFFERENT TEMPERATURES

As shown in Figure 5, with the extension of storage time, the titers of DSO stored at three different temperatures decreased. After three months, phage P1 stored at 4℃, -20℃, and -80℃ decreased by one logarithmic level. By the 4th month, the titer of phage P1 stored at 4℃ and -20℃ had been reduced by four logarithmic levels, while that of phage P1 stored at -80℃ had decreased by only two logarithmic levels, which was significantly higher than that of the other two groups. From the 4th month to the 10th month, the titer of phage P1 decreased gently. After ten months of storage, the titer of phage P1 decreased to 10^3 PFU/mL at 4℃ and 10^5 PFU/mL at -20℃ and -80℃. The protective effect of DSO against phage P1 at -80℃ was significantly higher than in other conditions ($P \leq 0.05$).

As shown in Figure 6, the titer of phage P2 decreased with the extension of storage time. The titer of phage P2 stored at -80℃ decreased by one order of magnitude after seven months and could maintain 10^7 PFU/mL after ten months. The titer of phage P2 stored at -20℃

decreased by one order of magnitude to 10^7 PFU/mL after six months, slightly lower than that of phage P2 stored at -80℃. The aging value of phage P2 stored at 4℃ decreased by one order of magnitude at four months, and the titer was 106 PFU/mL after ten months of storage, which decreased by two orders of magnitude. There was no significant difference in the preservation effect of DSO at -80℃ and -20℃ for phage P2 within four months ($P \leq 0.05$). After four months, the preservation effect of DSO at -80℃ was significantly better than the other two groups. Therefore, the optimum temperature of dimethyl sulfoxide as a protective agent for the preservation of phage P1 and P2 is -80℃, and the titer of phage P1 can be kept above 10^6 PFU/mL within four months, and the titer of phage P2 can be kept above 10^7 PFU/mL within ten months. In summary, the best preservation method for phage P1 is to preserve it at -80℃ with 15% glycerin as a protective agent. For phage P2, the best preservation method was 15% glycerol or 7% dimethyl sulfoxide as a protective agent at -80℃.

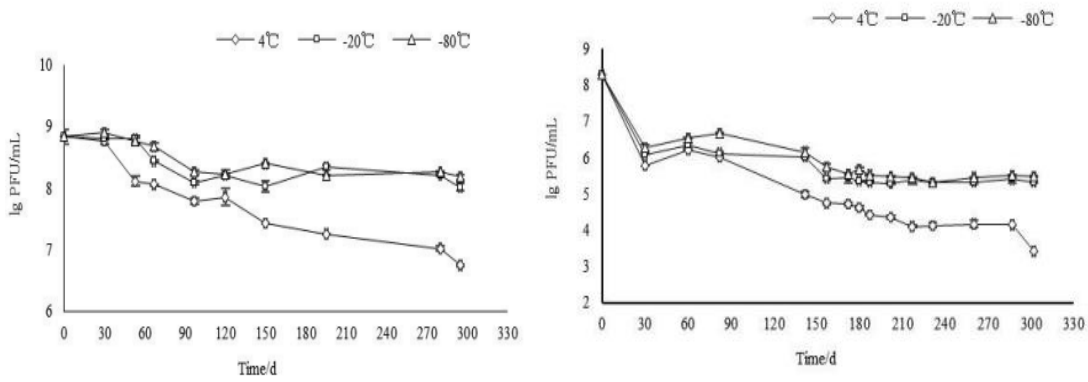


Fig. 5, 6 The phage P1 and P2 volume is stored in 7% dimethyl sulfoxide at different temperatures.

The results showed that phage P1 was stable at -20°C for 7 months at 10^5 pfu/ml, but decreased to 10^3 pfu/ml after 10 months at -80°C. Phage P2 is stable at 10^6 pfu/ml when stored at -80°C for 10 months. For 7% dimethyl sulfoxide, the optimum temperature for P1 and P2 phage storage is -80℃, P1 phage volume is more than 10^6 pfu/ml for 4 months, and P2 phage volume is more than 10^7 pfu/ml for 10 months.

4.2 TRANSCRIPTOMIC STUDIES OF PHAGE AT DIFFERENT STAGES OF HOST INFECTION, BASIC INFORMATION ON THE PHAGE TRANSCRIPTOME

To study the connection mechanism between phage and host bacteria, we selected phage P2 as a representative in our study. We plotted the one-step growth curve of phage according to Xi Yu et al., [29]. Drawing the one-step growth curve of the phage, it can be seen that the incubation period of the phage is 45 minutes, the lysis period is 45-135 minutes, and the stationary period is after 135 minutes. During this period, all the host cells have died. Since all host cells died during this time, in this study, only phage-digested host bacterial RNA was

extracted as the object of study at 0, 5, 15, 25, 80, and 120 min. Transcriptomic technology was used to comprehensively analyze the differential expression, gene regulation, and gene enrichment of phage P2 genes at different stages of host bacterial infection to investigate potential phage invasion and host bacterial lysis mechanisms.

In this study, we collected phage lysates of phage P2 at different lysis stages and used the Illumina Hiseq sequencing platform to complete transcriptome sequencing. An Illumina PE library for 2×150 bp sequencing was constructed, and quality control was performed on the obtained sequence data. Bioinformatic methods applied to the transcriptome data were analyzed, and the transcriptome size and GC content of phage P2 are shown in Table 1.

Table 1. Data statistic results

№	Number of sequences (strips)		Number of bases (bp)			Q30%	GC%
	raw	clean	raw	clean	raw	clean	clean
A0	1.19E+07	1.14E+07	1.78E+09	1.57E+09	92.88	95.35	42.38
A5	1.55E+07	1.51E+07	2.33E+09	2.10E+09	93.93	95.62	40.83
A15	1.99E+07	1.94E+07	2.98E+09	2.68E+09	93.65	95.55	41.14
A25	1.59E+07	1.55E+07	2.38E+09	2.11E+09	92.98	95.27	41.63
A80	2.55E+07	2.52E+07	3.82E+09	3.49E+09	95.66	96.76	42.04
A120	1.73E+07	1.67E+07	2.60E+09	2.30E+09	92.59	95.05	41.89

Note: raw represents the original sequencing data, clean describes the sequencing data after quality control; A0: initial lysis (0 min), A5: incubation period (5 min), A15: middle incubation period (15 min), A25: late incubation period (25 min)), A80: intermediate stage of lysis (80 min), A120: late location of lysis (120 min)

Table 1 shows the original and statistical sequencing data after quality control. It can be seen from the table that the actual sequencing numbers at the five points are all greater than 10 million, of which the number at 80 minutes is greater than 20 million, and the base numbers are all above 1568532099 bp. Q20% and Q30% are more significant than 92.59%, which meets the sequencing requirements. The average GC content is more critical than 40.83%.

Table 2. Mapping Ratio statistic results

Sample labeling	Sequencing number	Percent（%）
A0	7951913/11410320	69.69%
A5	3433221/15116980	22.71%
A15	3391843/19445526	17.44%
A25	3437022/15458148	22.23%
A80	4857998/25152682	19.31%
A120	3755291/16713974	22.47%

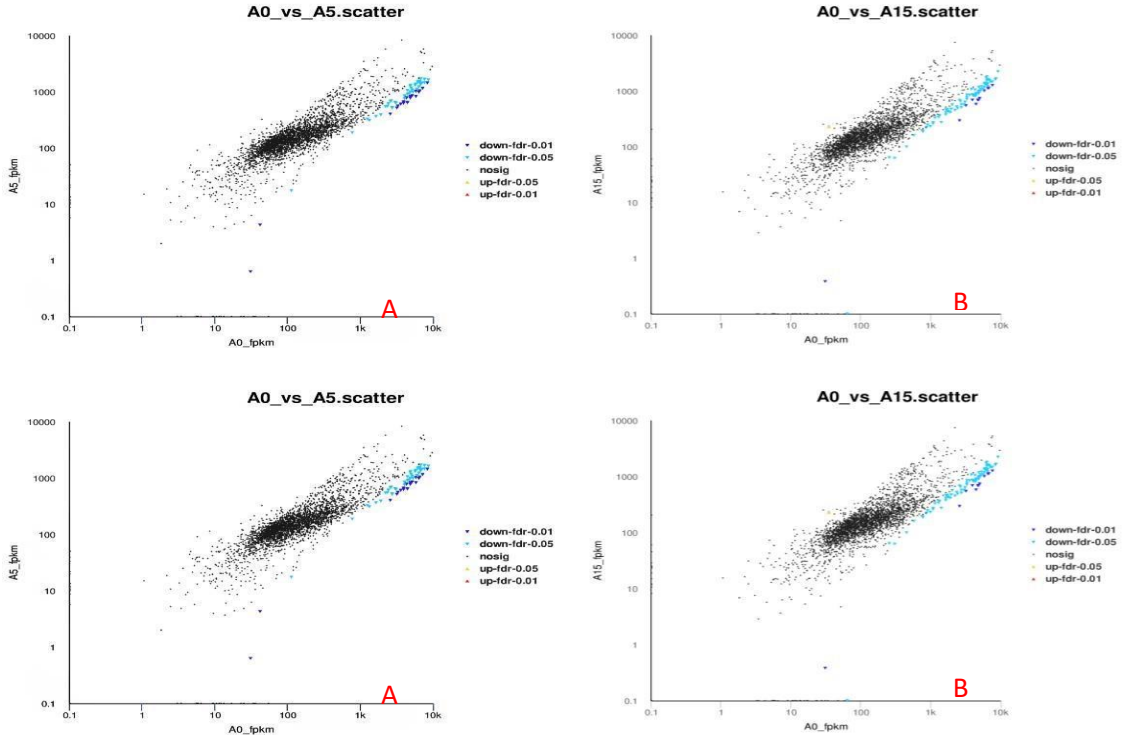
Note: A0: initial lysis (0 min); A5: incubation period (5 min); A15: middle incubation period (15 min); A25: Late incubation period (25 min); A80: Middle lysis period (80 min); A120: Late lysis period (120 min)

To obtain mapped data (reads) for further analysis, compare the clean, quality-controlled data (reads) with the reference genome. Table 2 presents the findings. There is no contamination in the relevant experiments and the reference genome used. Consistent with the sequenced species, the mapping percentage (mapping ratio) of clean reads on the reference sequence will be higher than 60%. In this study, phage P2 was selected at different time points when it infects host bacteria. The genetic material of the host bacteria will be changed when the phage integrates its genetic material or when incorporated into the genes of the host bacteria. As shown in the table, the comparison rate is greater than 60% at 0 min of infection, indicating that the sequencing data in this study are qualified and can be further analyzed and studied.

4.3 DIFFERENCES IN GENE EXPRESSION AT DIFFERENT STAGES OF PHAGE INFECTION

To understand the differences in gene expression at different lysis stages of phage, we used edgeR software to analyze the initial point of phage infection (0 min), the early incubation period (5 min), the middle incubation period (15 min), the late incubation period (25 min). Gene expression levels were sequenced at mid-lysis period (80 min) and late lysis period (120 min), as well as 0 min and 5 min, 0 min and 15 min, 0 min and 25 min, 0 min and 80 min, 0 min. Comparative analysis was performed with the differential genes between 120 min to guess the activity mechanism of the phage in different lysis periods.

The scatter-plots (scatter-plots) of 0 minutes and 5 minutes, 0 minutes and 15 minutes, 0 minutes and 25 minutes, 0 minutes and 80 minutes, and 0 minutes and 120 minutes are displayed in Figure 7.



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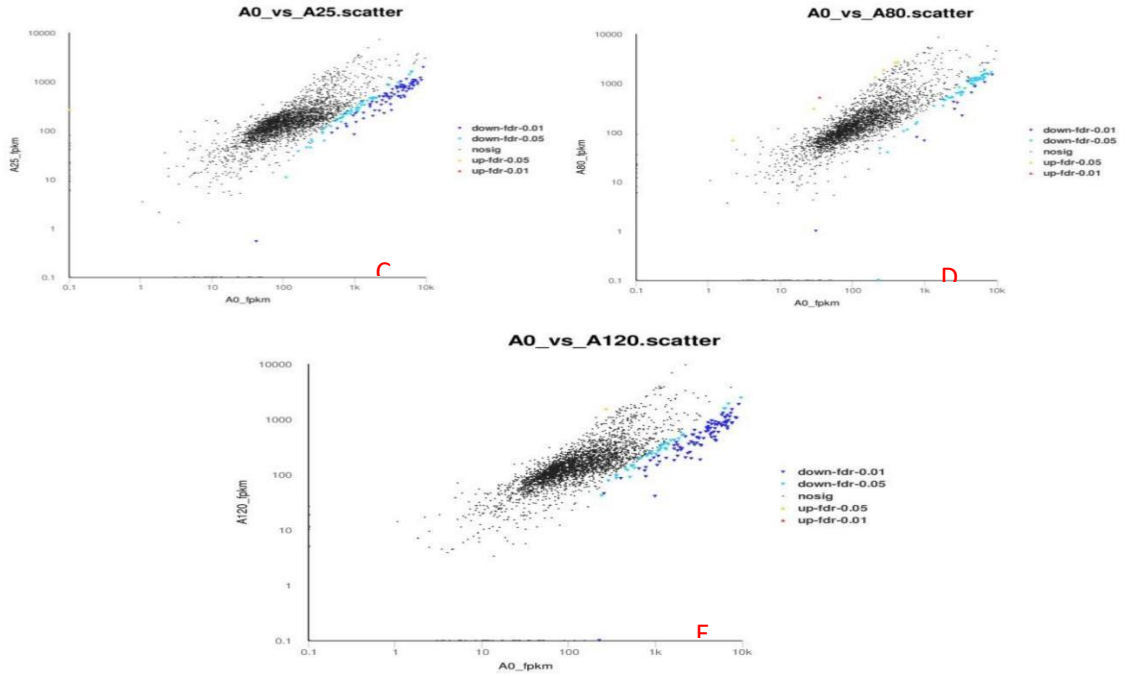

Fig. 7 Visualization of differential genes in different infection periods (scatter plot)

Note: A is a scatter plot of up-and down-regulated differential genes between 5 min and 0 min; B is a scatter plot for up- and down-regulated differential genes between 15 min and 0 min; C is a scatter plot of up-and down-regulated differential genes between 25 min and 0 min; D is a scatter plot of up-and down-regulated differential genes comparing 80 min and 0 min; E is a scatter plot comparing up- and down-regulated differential genes between 120 min and 0 min.

The figure shows the differential gene expression levels between the two samples. In the first stage of phage incubation (5 min), 65 genes were significantly changed compared to 0 min, and all of them were down-regulated, and in the middle stage of phage incubation (15 min), 92 genes were significantly changed compared to 0 min, of which 91 genes were down-regulated considerably and 1 gene was significantly up-regulated (gene 144); At the late phase of phage incubation (25 min), 143 differential genes were significantly changed compared to 0 min, of which 141 differential genes were down-regulated considerably and 2 differential genes were significantly up-regulated; During the lysis phase (80 min) compared to 0 min, there were 78 significantly changed differential genes, of which 70 genes were down-regulated considerably and 8 genes were significantly up-regulated; At late digestion (120 min) compared to 0 min, 144 differentially expressed genes were altered, 143 differentially expressed genes were significantly down-regulated, and 1 gene was up-regulated considerably (gene 2876).

4.4 FUNCTIONAL ENRICHMENT OF DISTINCT GENES AT VARIOUS STAGES OF PHAGE INFECTION

KEGG functional enrichment analysis was carried out on the differential genes in P2 phage infection stages to demonstrate the functional enrichment of differential genes and to ascertain the differences in genes in different stages of phage degradation. Fisher's exact test was utilized to determine the significance of the differences. Four multiple testing methods (Bonferroni, Holm, Sidak, and p-value) are used to correct the p-value to control the predicted false positive rate. This function is often highly enriched when the corrected p-value is less than the p-value ≤ 0.05.

Table 3. KEGG functional enrichment table of differential genes in different periods

	Pathway	Number of genes	P value	Function
0 VS 5	ko03010	39	5.10×10^{-30}	Ribosome
	ko00710	3	0.036611869	Carbon fixation in photosynthetic organisms
0 VS 15	ko03010	42	4.87×10^{-27}	Ribosome
	ko00710	4	0.018869624	Carbon fixation in photosynthetic organisms
	ko03070	3	0.041860584	Bacterial secretion system
0 VS 25	ko03010	47	2.22×10^{-23}	Ribosome
	ko00710	5	0.019871646	Carbon fixation in photosynthetic organisms
	ko03020	3	0.035799153	RNA polymerase
0 VS 80	ko03010	41	2.72×10^{-39}	Ribosome
0 VS 120	ko03010	51	3.30×10^{-28}	Ribosome
	ko00710	5	0.015315753	Carbon fixation in photosynthetic organisms
	ko03020	3	0.030220167	RNA polymerase

Note: A0: initial lysis (0 min); A5: incubation period (5 min); A15: middle incubation period (15 min); A25: Late incubation period (25 min); A80: Middle lysis period (80 min); A120: Late lysis period (120 min)

As shown in Table 3, during the initial incubation period (5 min) of the host bacteria phage infection, 39 genes were significantly downregulated in ribosomal metabolism compared to the beginning of the disease (0 min), while 3 genes were significantly downregulated in the carbon fixation pathway. When comparing the mid-incubation phase (15 min) of host bacterial infection to the early stage of disease (0 min), 42 differentially expressed genes were significantly down-regulated in the ribosomal metabolism process; additionally, four genes were significantly down-regulated in the carbon fixation pathway; finally, during the late-incubation phase (25 min) of host bacterial infection, 47 differentially expressed genes were significantly down-regulated in the ribosomal metabolism process. 3 genes were significantly downregulated in the RNA polymerase metabolism pathway; 41 differentially expressed genes were especially down-regulated in the ribosomal metabolism process during the lytic phase of bacterial infection (80 min) when compared to the early stage of disease (0 min) and 5 genes in the carbon fixation

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pathway were significantly down-regulated; 51 differentially expressed genes were significantly down-regulated in the ribosomal metabolism process during the late stage of host bacterial infection (120 min) when compared to the early stage of disease (0 min), and 5 genes in the carbon fixation pathway, and 3 genes in the RNA polymerase metabolism pathway were significantly downregulated.

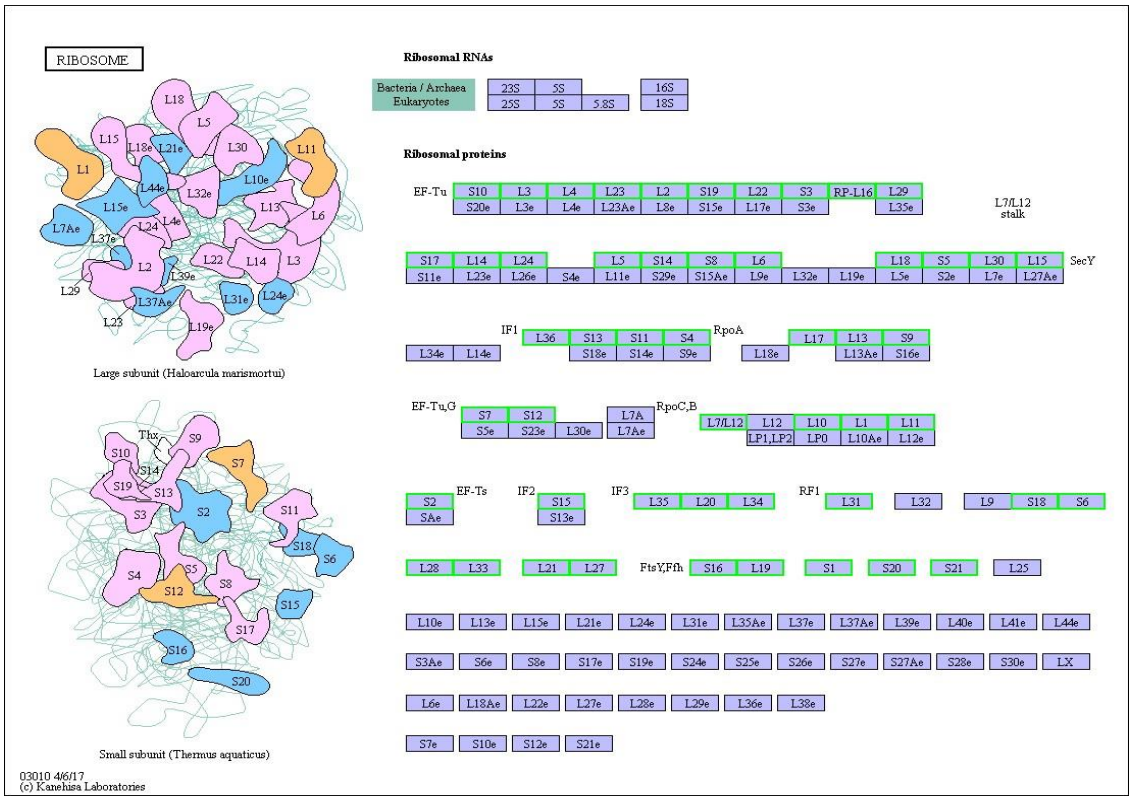

Fig. 8 Differential genes KEGG annotation pathway table of the ribosomal protein system

A KEGG-annotated pathway map of the ribosomal protein system's differential genes is shown in Figure 8. Genetic information processing and translation is the ribosome's main job. There is just one kind of ribosome found in prokaryotes, and it is made up of large and small subunits. The messenger RNA information is initiated when the ribosome starts the process of protein synthesis. The messenger RNA is joined by the small subunit, which subsequently interacts with the big subunit to form the complete ribosome.

During protein synthesis, EF-Tu and EF-G engage in interactions with ribosomes in this process. While EF-G aids in the forward elongation and translocation of messenger RNA and peptidyl-tRNA conjugates, EF-Tu transports amino acid-tRNA to the ribosome [30, 31]. Sec Y contributes to the co-information process in a supporting role [32], translation initiation factor F1 interacts with nucleic acids to start protein synthesis [33], cells lacking IF1 have nearly no polymer, which is critical for cell growth [34], and RpoA is a gene for RNA polymerase's α subunit. Through its interaction with an activator protein [35], which has two components that control bacterial transcription, it directly contributes to the activation of gene transcription. Phage P2 can be seen to disrupt bacterial transcription and information processing by controlling key enzymes such as IF1, RpoA, and EF-Tu in bacterial transcription and transcription processes.

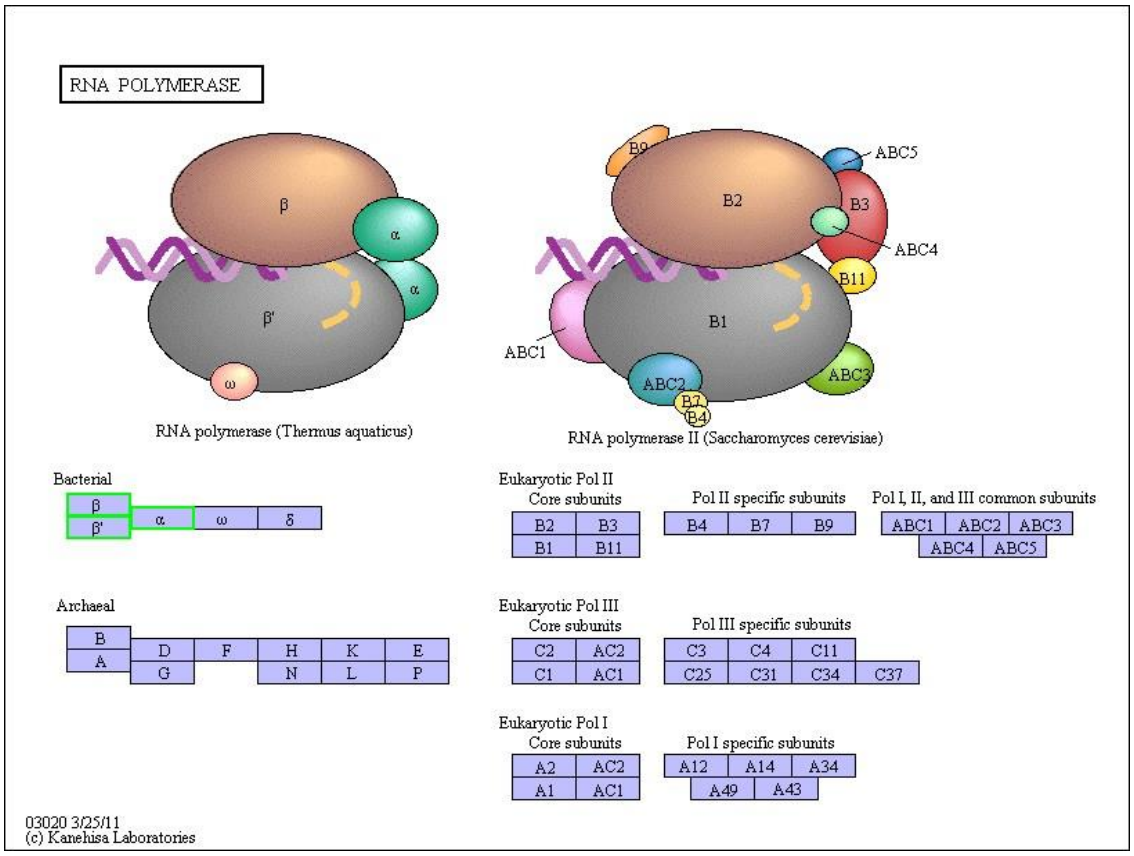

Fig. 9 KEGG pathway table of RNA polymerase differential genes

Figure 9 shows a diagram of the metabolic pathway of RNA polymerase. The primary function of RNA polymerase is to process genetic information and participate in transcription. It is an enzyme that catalyzes the synthesis of RNA from nucleoside 5'-triphosphate using a DNA or RNA strand as a template. The gene is markedly downregulated, as seen by the figure's green color. A β subunit, a β' subunit, two α subunits, an ω subunit, and a δ subunit make up RNA polymerase. α2ββ'ω is referred to as the key enzyme among them. It catalyzes the transcription and synthesis of all RNAs and is engaged in the transcription process in its entirety. The primary function of the δ factor is to identify the starting position of transcription, bind RNA polymerase to the promoter site, and recognize the promoter on the DNA template. It cannot exist separately. After binding to the template DNA and combining with the main enzyme to form a holoenzyme, the holoenzyme can be combined with a promoter on the template DNA. When it binds to a specific base sequence of the initiation gene, the double DNA strands are partially unwound, allowing transcription to begin. Factor δ is, therefore, also called the initiation factor. The genes that encode the α, β, and β' subunits of RNA polymerase are markedly downregulated when phage P2 infects a host bacterium for 25, 60, and 120 minutes. An increasing number of genes Through its interaction with activator proteins, the α-subunit of RNA polymerase is directly engaged in the activation of gene transcription, as demonstrated by examples [36].

5. CONCLUSION

P1 and P2 are virulent phages of Lactobacillus plantarum; studying the most appropriate method for their preservation is essential for the dairy industry. It is crucial for future research to use phages. It provides information for research, such as controlling the effects of bacteriophages, determining their biological properties, detecting phage-anti-strains, etc.

In this study, after ten months of long-term storage at 4℃, the optimal protective agent was dimethyl sulfoxide with a 10^6 PFU/mL titer. In conclusion, glycerin was the most suitable protective agent for preserving phage P1 at 4℃. At the same time, dimethyl sulfoxide was the most appropriate protective agent for the preservation of phage P2, and the preservation stability of phage P2 was better than that of phage P1 at 4℃. In addition, using 15% glycerol and adding 7% DMSO at -20℃ within 112 days had an excellent preservation effect on phage P1, and its titer could be maintained above 10^6 PFU/mL. Therefore, glycerol and dimethyl sulfoxide are the most suitable protective agents for phage P1 at -20℃. When phage P2 was stored at -20℃, glycerol, chloroform, and dimethyl sulfoxide could be used as protective agents within four months, and their titers were maintained above 10^8 PFU/mL. After four months, the best protective agent for long-term preservation was 15% glycerol, which could keep the phage titer above 10^7 PFU/mL. It was also found that the titer of phage P1 decreased by two logarithmic orders after being preserved for four months by adding 15% glycerol or 7% DSO at -80℃. After ten months of storage, the titer of phage P1 could still reach 10^5 PFU/mL. Therefore, glycerin or dimethyl sulfoxide was the best protective agent for phage P1 stored at -80℃ for four months. When phage P2 was stored at -80℃, 15% glycerol or 7% dimethyl sulfoxide was the best protective agent, and its titer could maintain 10^7 PFU/mL after ten months of storage. It can be seen that -80℃ is suitable for long-term storage of phage, but for phage P1, storage at -80℃ also needs to be transformed every four months to maintain its vitality. Pnagus P2 showed higher activity than P1.

This transcriptomic study of phage P2 infecting L. plantarum IMAU10120 at different stages showed that, compared with the initial point of infection by the phage, the encoding elongation factors EF-Tu, EF-G, and EF in the latent period, mid-lytic period and late lytic period -Ts, the synthesis of Sec Y protein, prokaryotic translation initiation factors IF1, IF2, IF3, RNA polymerase Rpo A, Rpo B, Rpo C, signal recognition particles Fts Y, Ffh and release factor RF1 was significantly reduced, and phage P2 passed. It regulates critical enzymes of the ribosome and RNA polymerase metabolic pathways to complete its genetic material's replication, transcription, and translation. It simultaneously inhibits the transcription and translation of bacterial genetic material. RNA polymerase synthesis decreased significantly when virulent phage P2 infected Lactobacillus plantarum IMAU 10120 for 25 min. Therefore, we speculated that phage had completed the transcription and translation process of its genetic material, that is, the assembly process of phage. However, when phage lysed for 80 min, The RNA polymerase resumed its original function, and we speculated that phage might produce some enzymes that lyse the bacteria's cell wall during this period.

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Current development of ESP teaching policies in Asian countries and at public universities in Mongolia

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Current development of ESP teaching policies in Asian countries and at public universities in Mongolia

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Abstarct- With the further expansion of economic globalization, more and more joint ventures and foreign-funded companies have been established and have made significant contributions to the world market economy. All fields increasingly demand inter-disciplinary talents who are skillful in their major as well as foreign language. Society tends to demand for one's foreign language ability to contain level of diversity and specialization. For instance, those who major in trade can fluently use English to participate in business negotiations, those who major in news can use English to interview, those who are layers can use English to engage in various cases, and those who are engineers can use English to communicate with foreign technicians. Therefore, it is hard for those who have merely a general ability to be best suited for market demands. Mongolia's foreign relations are rapidly developing, investments in the business and mining sector are increasing, and the need for ESP with high English skills to manage those activities is increasing dramatically. In line with this, we aim to contribute to the development of ESP by studying the ESP policy and the characteristics of ESP training in Asian countries with similar cultures to our own country.

Keywords- English language policy and ESP, ESP in Asian countries, ESP at public universities in Mongolia

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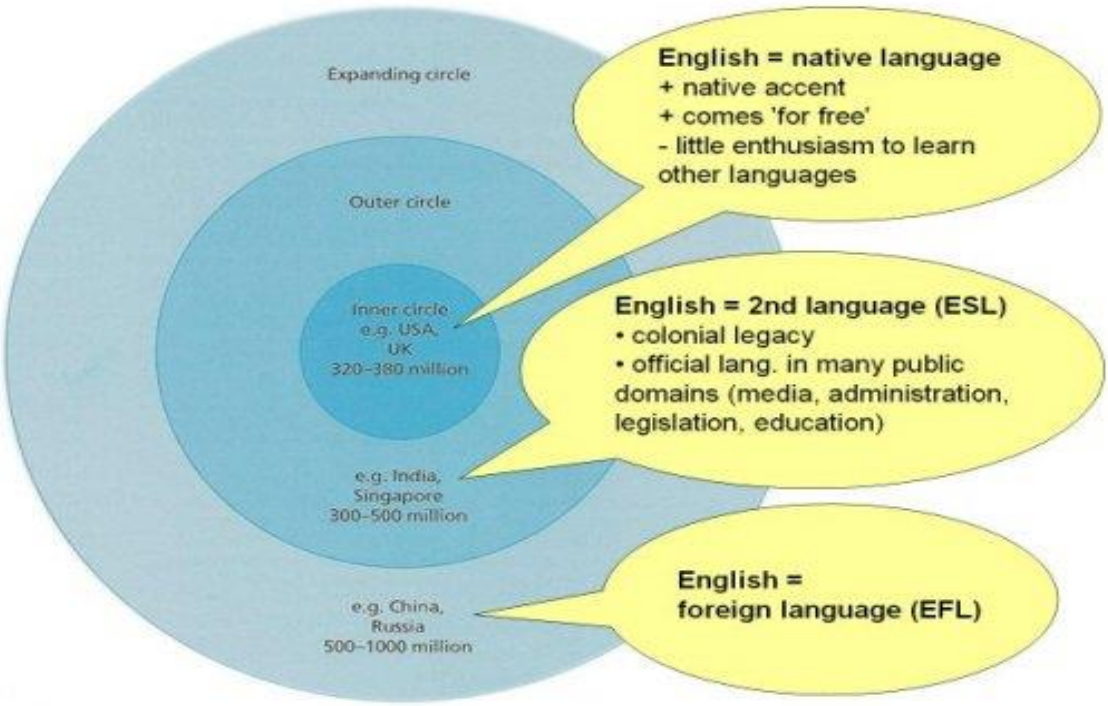
I. INTRODUCTION

English for specific purposes (ESP) refers to the teaching and learning of English as a second or foreign language where the goal of the learners is to use English in a particular domain [1]. ESP has come a long way in terms of research practices since its inception in the 1960s, but genre, a topic initiated in 1981, remains with us. In addition, other topics and methodologies have opened the way for leaner centered approaches, advocated by Hutchinson and Waters [2] , and later, by Tardy [3]. This has now expanded to include other areas such as English for academic purposes (EAP), English for occupational purposes (EOP), English for vocational purposes (EVP), English for medical purposes (EMP), English for business purposes (EBP), English for legal purposes (ELP), and English for sociocultural purposes (ESCP) [4]. There will probably be further interest in classroom-based research and in studies in less-popular academic locales, such as secondary and vocational schools or in regions where English is the lingua franca. Perhaps, unfortunately, for the needs of local students and international scholars, research may become more centralized in international journals, though online publications may mitigate some of these issues [1].

II. THEORETICAL OVERVIEW

The most common classification of English, especially in the language teaching world, has been to distinguish between English as a native language (ENL), English as a second language (ESL) and English as a foreign language (EFL). Kachru [5] however, put forward another classification outlining the roles of English in the world as three concentric circles: the inner circle, Outer circle and expanding circle. The Inner Circle refers to countries where English is used as a mother-tongue language for example, USA, UK, and Ireland. The outer Circle refers to countries where English is used as a second language for example, Singapore and India. The expanding circle refers to countries where English is used as a foreign language or an additional language, for example Thailand and China. When we study language policy and ESP teaching policy, we have chosen Asian countries which included in expanding circle. For example, China, Taiwan, Japan, Spain and Mongolia. The main aim of this research is to study ESP teaching policy in Asian countries, take example of ESP teaching and curriculum developing method, make comparison with Mongolia’s ESP teaching policy.



Fugure 1. Kachrus three circle model

Needs analyses, carried out to establish the "what" and the "how" of a course, is the first stage in ESP course development, followed by curriculum design, materials selection, methodology, assessment, and evaluation. However, theses stages should not be seen as separate, proceeding in a linear fashion. Rather, as noted by Dudley-Evans and St John [6], they are interdependent overlapping activities in a cyclical process. A broad, multi-faceted definition of needs analyses is provided by Hyland [7].

III. SURVEY OVERVIEW

The main task of the study was to determine ESP future developments trend in Mongolia carrying out current development of ESP influence of universities in Asian countries. The study was carried out over a period of four years from September, 2020 to June, 2024.

The objectives of the study are:

(1) To identify ESP teaching policy, training features at public universities in Mongolia
(2) To determine what initiatives school leaders initiate to create school climate for promotion of education for sustainable development in culturally diverse schools.

To accomplish the proposed goal, the study raised three research questions:

(1) What are the main characteristics of ESP curriculum, textbooks, teaching materials and teaching staff of ESP in Asian countries that have same culture as our country?
(2) What are the features, curriculums, textbooks, teaching materials and teaching staff of ESP at public universities in Mongolia?
(3) What to do to determine the future direction of the development of ESP at universities in Mongolia by studying the similarities and differences of ESP curriculum, textbooks, teaching materials and teaching staff of universities in Asian countries.

Composition of the Research Team

The team consists of 8 members – three ESP experts and five graduate students earning their degrees in educational studies. First, all the team members have been trained to build a common understanding of ESP. The team is divided into two groups: some focused on the observation of ESP training process at universities in Mongolia and the others analyzed ESP curriculum, textbooks, teaching materials and teaching staff.

Methodological Overview

The distinctive methodological characteristics of the study are:

1. Focus group interview was conducted with ESP teachers and a questionnaire with 12-questions was taken not only from ESP teachers but also ESP students. Also needs survey was taken from the ESP students.
2. Research was conducted on textbooks, teaching materials and teaching staff at public universities in Mongolia.
3. Studied the characteristics of ESP in Asian countries and determine the future direction of ESP in our country.

The Study and Data

The main study is concerned with a comparative study on current implementation of ESP policy in Mongolia and ESP training trends in Asian countries. With the emphasis in this study it is intended to analyze actual ESP practices in some Asian countries included in outer circle and in an expanding circle for the scope of English language and tried to compare their ESP training features and policy. Different types of articles through internet sources were used for data procession. The results of quantitative and qualitative analyses were combined and conclusion was drawn. 5 countries from the Asian countries included in outer circle of the scope of English language and 3 Asian countries included in an expanding circle for the scope of English language ESP teaching features were analyzed, but we focused more on China, Japan and Taiwan where ESP training has been developing rapidly.

Table 1. Comparative research on ESP in Asian countries and role of English

Countries	ESP policy and training features
Countries learning English as a second language	
India	• Some scientific courses are taught in English • English is important and also plays a role in ranking
Vietnam	• Some universities have a policy of teaching some courses in English • The policy is to use English in the profession • Books compiles in Canada are used for English language courses and teachers are trained in Britain.
Malaysia	• Malaysia has a vision to reach international communication technology (ICT) by 2025 and main key to reach this is considered English language training (ELT). • For all levels of schools Math and Science courses are being taught in English starting from 2003.
Singapore	• English is considered as a first language. • English has been identified as the main language of international workplaces. • All courses are taught in English besides native language.
Taiwan	• The country with the best English language courses in Asia • There is a competition between English teachers and subject teachers
Countries where English is studied as an international language	
China	• New model of dual vocational training “Major+English” • Business English has been approved as a compulsory subject by the Ministry of Education starting from 2007
Korea	• English as understood as an international language • English is believed the most important subject than other courses
Japan	• Program named “Japan with English ” implemented in 2007 • Model named 4-4-4 to improve general English and ESP • ESP community with 28 members.

This study did not evaluate learning outcomes of students in terms of ESP and therefore, only focused on observations and analyze of ESP actual educational practice in classrooms and universities.

Sample of Selected Schools

Team members discussed and 3 top public universities in Mongolia were selected to visit and to observe actual educational practices in the area of ESP. These are MNUE (Mongolian National University of education) where the researchers and teachers who made this research work, MNUD (Mongolian National University of Defense), and MNUMS (Mongolian National University of Medical Sciences).

Research conducted from 2020-2024 and 3 public universities, 909 bachelor students, 104 master students and over 50 ESP teachers included in our survey. From the public universities we will focus on *MNUE (Mongolian National University of Education),* where we teach.

Data Collection Instruments

The following instruments were developed to obtain standardized baseline data on the mainstreaming of ESP in Asian countries and at universities in Mongolia:

- Microscopic analysis (observations, survey, interviews on actual practice):
  i. Classroom observation instrument: 8 pages
  ii. Guideline for expert team report: school visit – 5 pages

The instruments for the analysis of Chinese universities the researchers developed a model for ESP teachers in China and determined 7 stages of routes of disciplinary development of business English as a sub-brunch in China. Most of the international items in these research instruments are built upon the following well-known studies and frameworks of ESP:

- English as A Lingua Franca in the international University Toolkit [4]
- World Values Survey 7 (WVS 2017-2022)

The study on textbooks and training materials were conducted from 2020-2024. First an evaluation sheet was developed for 18 ESP textbooks compiled by ESP teachers of English Department at MNUE (Mongolian National University of Education). To develop a textbook evaluation sheet a handbook compiled by UNESCO “Education for Sustainable development” and “A joint research report for ESD” conducted in Asia Pacific region were used as a methodological recommendation. Research on books and handbooks of ESP at MNUD (Mongolian National University of Defense) was analyzed to compare it with our university ESP textbook and handbooks and to determine specific features of ESP.

Also, a gender survey and a citizenship survey of students participated in survey were conducted. 13 ESP curriculum developed by ESP teacher s and difficulties for ESP teachers and their degree of education, employment years at MNUE were analyzed. Research on ESP curriculum of the Mongolian National University of Medical Science was analyzed to compare it with our university ESP curriculum and to determine specific features of ESP.

Besides of students needs analyzes research on improvement of ESP training was conducted from ESP teachers based on inquiry and continuous improvement criteria.

IV. RESULTS

China- With the development of China's economy and the enhancement of the country's internationalization, ESP has developed to some extent. To meet the needs of the market-oriented economy, various English training courses are offered, for example, foreign trade English, tourism English, English secretary, international finance and so on. About two decades ago, universities and colleges began to offer English courses concerning students' specific professional needs.

However, there is not yet a sound ESP system which is suited to the specific situation of China's higher education. In College English Syllabus, it has been stated that the ESP course should be a compulsory course; in the first two years of the undergraduate study, students are offered the course of Basic English; ESP should be set from the fifth to seventh semesters with no less than 100 hours altogether and two periods each week is required. If time or resources permit, it is better to offer EAP reading, ESP translation or ESP writing courses on the eighth semester. According to the survey conducted by the Foreign Language Department, Henan University, 58% of institutions of higher learning questioned failed to set up the ESP course as regular course due to lack of finance, equipment and teacher resources. So we can see that ESP develops very unevenly in China.

At present, business English in China is highly recognized in the society as one of the most popular disciplines. Statistics shows that the number of universities that have opened Business English major courses exceeds 800, and the schooling levels, majors and degrees are more diversified. For example, in Guangdong University of Foreign Studies, there are already five undergraduate programs and four postgraduate programs. Include the status of disciplinary development, course design, teaching approach, teaching staff development, and student evaluation system, which are seen as follows:

In China today, Business English evolving from ESP has developed into a formalized discipline the concept of register analyses in recent years, which forms a brand-new innovative way to the development of ESP different from other countries. (See Table2)

Table 2 Route of disciplinary development of business as a sub-brunch in China.

Stage1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage 7
Register analyses	Discourse analyses	Target situation analyses	Skills and strategies analyses	Learning-centred approach	Business English major	Business English discipline

In general, Business English evolving from ESP has a development history of over 50 years 7 stages in China.

Due to the different teaching or learning objectives, the courses of Business English for undergraduate or postgraduate programs have been designed with a general principle of approximately 70% of English language courses and 30% of business theories and practices such

as introduction to Business, Introduction to Economics, International trade, Business communication, International finance, International settlement, etc.
For the teaching approach single English teaching and bilingual teaching tends to be smaller and smaller in China. Besides simulation teaching, task teaching, interactive teaching methods can be very applicable in business English. Type of multi skilled teacher can be devided into three basic categories. (See table 3)

Table 3. Three basic types of multi-skilled teacher team proficient for business English in China

	Type	
1	Teachers of English major+ business management major	This type of business english teachers has two majors, who can teach both English courses and business management courses and conduct academic researches in both the fields of study.
2	Teachers of English major+fairly good business management knowledge base	This type of business English teachers has an English major, who can mainly teach English courses and conduct academic researches mainly in the linguistic field of study.
3	Teachers of business management major+ fairly good English language base	This type of business teachers has a business management major, who can mainly teach business management courses in English, and conduct academic researches mainly in the field of business management.

Taiwan- Today, an effective English program must meet both students' academic and career needs. Universities in Taiwan, in keeping with global trends, have begun to incorporate academic language skills, which students need to cope with English-taught programs in their majors, into English instruction (Hu, Chen & Liu 2008, cited in Tsou, 2009:78). Tsou (2009) describes the structure of the program at NCKU as follows: National Cheng Kung University successfully transformed sophomore English courses into an ESP format, helping students develop academic and professional skills. Thus, starting the fall of 2009, first-year students enroll in the EGP-based Freshman English, and in the second year, ESP-based sophomore English. In the same year, the program launched 10 new ESP sophomore English courses to replace the general classes. The new program included courses for liberal arts, management, medicine and science and engineering students. For each discipline, the program offers at least two courses. Management and Liberal arts students can enroll in Economics English and Business Management English. These are EAP courses. Or they can register for Careers English or Tourism and Hospitality English which are pre-service EOP courses. For science and engineering sophomore students, the program offers EAP courses on General Science, General Engineering, Hi-Tech industries, and Information Technology. Students of the medical college can attend Bio-technology and Bio-medicine, which are also EAP in nature (Tsou, 2009:78) At national universities , ESP is not a problem for two major reasons: first, these institutions can obtain funding to hire ESP teachers, and second, there is no conflict between the ESP teachers and the professors, as the latter are not interested in teaching ESP courses. In terms of expertise and perceived ESP teacher identity, both

language teachers' and subject teachers' are legitimized to teach ESP, equipping students with language competence and knowledge to participate in international discourses and practices. Therefore, a heated debate over "Who should teach ESP" has arrived at an ill time, when both both language and subject teachers have to fight over curriculum control to secure academic positions, due to Taiwan's recent student shortage.

Japan – For the language policy to promote an improvement in Japanese people's communicative ability in English was translated in 2002 into the "Strategic plan to cultivate 'Japanese with English abilities.

Japan Association of College English teachers organize ESP meeting every year. As the need for ESP in tertiary English education rises globally in tertiary education, a new English preprogram called "Technical English" was developed at a Japanese university of science and technology in Tokyo. This first university-wide compulsory ESP program at national universities in Japan was put in action in the academic year of 2012.[†] A closer examination of the academic genres selected by the whole science faculty for the new curriculum, such as academic presentations using ppt and posters, oral reports of academic articles and original research, abstracts, research papers, will be conducted in this study.

ESP program at Toyohashi University of Technology, this is a short-term exchange program with the Institute of Technology, Bandung, in Indonesia. Teachers make program's background and outlines, followed by a comparative analysis of students' comments before and after participating in the program. Issues and challenges are discussed, with the hope that the findings and implications will prove useful for improving ESP exchange programs further.[‡]

Different skills are taught for ESP students. For example, Summary writing is an essential skill for science and technology students. Students often need to read and understand scientific literature and then summarize the contents of the articles into a short paragraph. Summarizing the contents require extensive paraphrasing skills of the student. However, in the teaching of academic English in Japanese universities, it is not a skill that is given much importance. Teaching of summary writing to third-year undergraduate students in a technical English course at a Japanese university of science and engineering was successfully achieved.

Even in JACET (the Japan Association for College English Teachers) there are few specialists in ESP out of more than 2300 members. There is a small but significant movement, which is promoting ESP in Japan.

Mongolia-Research conducted from 2020-2024 and 3 public universities, 909 bachelor students, 104 master students and over 50 ESP teachers included in our survey. From the public universities we will focus on *MNUE (Mongolian National University of Education),* where we teach.

[†] SHI Jie (English Department, UEC Tokyo), JACET ESP meeting, 2014

[‡] Yo In'nami is an Associate Professor of English at Shibaura Institute of Technology, JACET ESP meeting, 2012

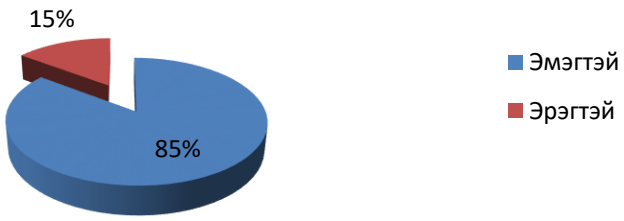


Figure 2. Gender of undergraduate students

For the gender of undergraduate students 85% of the students are female and 15% of the students are male, which means female students are being prepared to be future teachers at different subjects.

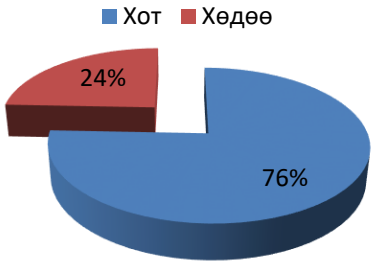


Figure 3. Citizenship of undergraduate students

For the teacher training university 76% of the students are from province and 24% from capital city, which means necessity of teacher's workplace is high in province than in the city.

Table 4. Research on ESP curriculum at MNUE

Subjects	**Number of curriculum**	**Level of language**	**Year**
School of Humanity: (history, Philosopy, Culture, Tourism)	4	Intermediate	2022-2023
Information technology, Math	2	Intermediate	2023
Art and design	1	Intermediate	2023
Literature	1	Intermediate	2023
Journalism	1	Intermediate	2024
Physics	1	Intermediate	2020
Physical culture	1	Intermediate	2020
EAP	2	Upper intermediate	2024
Total	**13**		

Table5. Difficulties for the English teachers of MNUE

Low degree of General English knowledge	72%
Lack of ESP standard and curriculum from the government	60%
High amount of students in a class	52%
High percent of teaching hour	24%
To improve ESP books, textbooks and materials	20%

Table 6. Why ESP teaching is necessary for students

to gain necessary information and put into practice	24%
To gain education to meet worldwide standard	16%
To get a job	6%
To work in international organization	1%
To improve knowledge	1%

ESP teacher's degree of education and employment years

Most of English teachers 75.8% at MNUE still studying at doctoral courses. Reason of this is lack of experienced supervisor and they have much of teaching hours than conducting any survey. 17.2% of the teacher's have master degree and 2% of them are doctor Ph.D or vise professor. 65.5% of ESP teachers employed over 16 years, which means experienced teachers; 24.2% of them employed 11-15 years, 10.3% employed 6-10 years.

Table 7. Research on books and handbooks of ESP at MNUE

Name of the books	**Level of language**			**Number of handbooks**
	Pre-intermediate	**Intermediate**	**Upper-intermediate**	
English for Social sciences and Humanities	2	2		4
English for Math and IT	1	2		3
English for art and design	-	1		1
Foreign language	1	2		4
English for literature	-	1		1
English for natural sciences	-	1		1
English for physical sciences	-	1		2
EAP	-	2	2	2
Total	1	5	12	18

Table 8. Research on improvement of ESP training

№	Index	1	2	3	4	5
1	Curriculum content should meet social necessity	1	0	0	1	18
2	To process ESP	1	1	2	15	2
3	To improve ESP books and handbooks	4	10	5	1	1
4	To make ESP as a compulsory subject	9	4	7	1	21
5	To renovate ESP teaching method and to improve information communicative technology	8	4	4	2	3

In Mongolian job market, there is an increasing demand for talents who not only possess all the necessary expertise, but also a good command of English. Therefore, the need for training compound talents has become increasingly prominent. For MNUE students, program named "Teacher with English abilities" has been implemented by the 70% of government scholarship to promote future teacher and develop the nation. This program at MNUE usually studying procedure as follows: the freshmen or sophomore, who has intermediate degree of English are entitled to applying for admission to above mentioned program with duration of two years. Students take advantage of winter holidays to pursue the above program. There are four compulsory courses in each semester within two years, with practical courses completed in the winter and summer vacations.

Mongolian National University of Defense- For the books and textbooks ALC (American Language course) or military English books have been used for over 20 years not only for University of Defense students, but also for all military units and branches. These books are delivered to all collaborative countries from DLI (Language Institute of Defense) within IMET (International Military Education and Training) program of USA. These books have 5 levels each consists of 5 sections and Military English is taught 3 credit hours for a semester.

Table 9. Research on books and handbooks of ESP at MNUD

Levels of the books	Level of Language	Points to be meet
1-6	Elementary	0-25
7-12	Pre-intermediate	25-50
13-18	intermediate	50-60
19-24	Upper-intermediate	60-70
25-34	Advanced	70-80

From the study we can conclude that for the University of Defense ESP teaching policy to meet an international standard has been implemented.

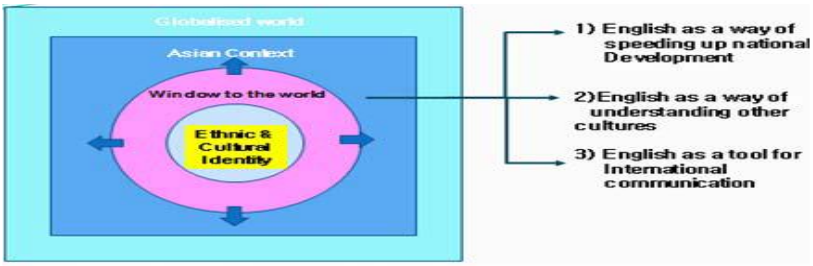
Mongolian National University of Medical Science- For the MNUMS from 2010 a little bit changed ESP teaching policy and in total 8 credit hours of English is taught for freshmen and sophomore. 4 credit hours are for General English in 5 levels from elementary to advanced level. Other 4 credit hours of ESP or English for medical science is taught for sophomore.

Table 10. Research on ESP curriculum of the Mongolian National University of Medical Science

Subjects	General English	Credit hours	ESP			
	Form of the subject	hours	Form of the subject	Cr hours	Form of the subject	Cr hours
Traditional medicine	Compulsory	2	Compulsory	2	Self – study	2
Bio medicine	Compulsory	2	Compulsory	2	Self – study	2
Social health	Compulsory	2	Compulsory	2	Self – study	2
Head& face medicine	Compulsory	2	Compulsory	2	Self – study	2
Treatment	Compulsory	2	Compulsory	2	Self – study	2
Nursery	Compulsory	2	Compulsory	2	Self – study	2

From the study we can conclude that at Mongolian National University of Medical Science ESP teaching policy is different from other universities in its form, 2 credit hours are compulsory and 2 credit hours of ESP are in the form self-study.

As a conclusion of this study , the essential role of English language education in Asian context can be summarized as Figure 1. **(Chang, .B-M. 2011). The roles of English language education in Asian context. *Journal of Pan-Pacific Association of Applied Linguistics, 15* (1), 191-206.)**



V. CONCLUSION

In conclusion, the ESP course can be considered as the most practical and applicable subject for universities in Asia as the students want to become successful learners in their professional sphere while learning English. In China business English is highly recognized in society and there has been aroused a competition between subject teachers and ESP teachers, in Taiwan different kinds of ESP courses for sophomore, in Japan universities different ESP programs have been implemented. Mongolian ESP teachers work mastering both in linguistic and specialized areas, the ESP standard for Mongolian university academic programs, which theoretical and practical fundament has already been established firmly by the university ESP teachers initiation and efforts, should be formulated and brought into the wide implementation at the tertiary education. The process of transmitting from general English to ESP in public universities in Mongolia has a trend of rapid development. ESP teachers at public universities in Mongolia develop ESP curriculum based on the needs of students and also compile ESP textbooks based on the above curriculums. ESP teachers at public universities in Mongolia work as a team to develop a ESP curriculum based on the needs of students. After being approved by the sub-committee of the curriculum and then by the subcommittee of branch school, teachers are allowed to teach ESP. Some ESP teachers at public universities in Mongolia work together with teachers from professional departments to develop ESP curriculum, and in the future , there may be competition between English ESP teachers and professional teachers from professional departments.

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The study of normative levels of indicators for the company's assets and capital structure

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The study of normative levels of indicators for the company's assets and capital structure

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Abstract: One of the primary challenges in analyzing a company's financial statements is establishing the normative level for financial ratio indicators. However, in our country, there lacks a unified methodology for determining these normative levels based on the business sector and company size. Therefore, the goal of this research is to assess the normative level by using working capital as a representation of the capital structure and the debt ratio as a representation of the capital structure. In our study, we analyzed a total of 2,200 samples of companies listed on the Mongolian Stock Exchange (MSE) between 2009 and 2022. Based on our research findings, the debt ratio and the percentage of working capital exhibit a non-linear relationship with profitability indicators. It is advisable to establish the normative level as a median rather than an average value, as normative levels also vary depending on factors such as company size, industry, and economic growth.

Keywords: Debt ratio, Return on capital, Normative level, Working capital, Joint stock company

1. INTRODUCTION

In the corporate sphere, comprehending the optimal allocation of the company's assets and capital structure, and ensuring the appropriate equilibrium between debt and equity, holds

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paramount importance in assessing financial health indicators and fostering sustainable future growth [1]. Until now, in our country, there is no unified methodology that determines the normative level of financial ratios depending on the business sector and the size of the company. Therefore, we set the following goals with this research work:

1. Assess the normative level by representing the percentage of working capital representing the capital structure and debt ratio by representing the capital structure
2. To study the relationship between the mean and median of these indicators and the profitability indicators

The stable and profitable operation of the company yields a positive impact not only on the organization itself but also on all stakeholders, including investors, customers, and employees [2]. The primary indicators that can determine and evaluate the current financial situation of the company are the current ratio and the debt ratio. The current ratio assesses the organization's ability to meet its short-term obligations with its current assets, while the debt ratio calculates the percentage of external financing in relation to the total assets [3]. A company's survival is contingent upon working capital, which serves as a critical resource for financing and decision-making throughout the operational cycle, spanning from the procurement of raw materials to the production of final products [4]. Short-term liabilities are typically settled using the cash generated from working capital, and reducing the company's liabilities has a positive impact on financial stability and enhances liquidity [5]. Financial managers and executives prioritize determining the appropriate level of working capital and implementing optimal management methods to ensure sufficient resources and cash to cover short-term liabilities and future planned operations [6].

Establishing normative levels for specific indicators related to the company's assets and capital structure is a common practice aimed at ensuring financial stability, efficient operations, and optimal risk management [7]. These norms can vary across countries and are typically established by regulatory bodies or industry standards. Economically developed nations like the United States, the United Kingdom, and Japan rely on standard norms developed by top institutions for financial stability regulation. They evaluate changes and efficiency in reporting years for branches and organizations, using these evaluations to develop strategic policy plans for the future.

In developed countries, particularly in our nation where the profitability of companies is pivotal amid unstable economic fluctuations for economic growth and development, various regulatory frameworks are employed to ensure financial stability, transparency, and comparability of financial reports. However, unlike evaluating based solely on financial analysis and historical reports, it is challenging to establish normative levels for parameters related to capital and capital structure of companies through standard-setting organizations. Hence, there exists a challenge in establishing normative levels that enable financial comparisons based on factors such as the sector, scale of operations, and asset conditions.

2. THEORETICAL BACKGROUND

An optimal capital structure refers to the resources a company utilizes to finance its assets, operations, and future growth, with financing sources typically encompassing debt and equity. The primary theories in this domain include Modigliani and Miller's theory, trade-off theory, and pecking order theory, all of which aim to elucidate how the departure from normativity influences a company's capital structure [8].

The study of optimal capital structure gained significant popularity following the introduction of Modigliani and Miller's theory [9]. The Modigliani and Miller theory posits that there is no distinction between the utilization of debt and equity and that the composition of a company's capital structure, whether it includes debt or equity, does not impact the firm's value. Additionally, the theory develops propositions I and II. [10]. Proposition I of Modigliani and Miller's theory discusses the impact of taxation, while Proposition II highlights that firms with higher debt financing experience a higher cost of equity capital. In essence, these propositions elucidate how debt influences both the value of a firm and the cost of equity capital. It's important to note that in a perfect market, the capital structure does not affect the firm's value, but in reality, imperfections such as taxes come into play and affect the value of the firm.

The trade-off theory posits that companies aim for an optimal capital structure that strikes a balance between the tax advantages of debt and the costs associated with debt. According to this theory, firms borrow funds until the tax benefits derived from debt outweigh the costs associated with potential financial distress. Empirical research supports this theory by showing that firms endeavor to minimize their weighted average cost of capital (WACC) through prudent management of their debt levels. [11]. Companies strive to enhance value by leveraging debt to minimize tax liabilities and augment cash flow [12]. Non-debt tax shelters provide additional tax advantages such as depreciation and diminish the necessity for debt. Research indicates that companies with greater non-debt tax shelters tend to have lower levels of leverage [13]. There is a positive correlation between the value of real assets and leverage, suggesting that real assets are more readily used as collateral for obtaining loans [14]. The capital structure of Dutch companies aligns with the trade-off theory, as it involves a careful balance between maximizing tax benefits and managing the complex costs associated with debt [15].

Excessive debt elevates the risk of financial distress, which can lead to bankruptcy and associated costs [16]. These costs encompass legal and administrative expenses as well as missed business opportunities. The fluctuation in operating income signifies business risk, with high volatility typically linked to low leverage [17]. Larger companies generally face fewer difficulty costs, making debt financing more accessible for them. It can be inferred that the leverage decisions of Dutch companies are guided by the trade-off between tax benefits and difficulty costs, which are influenced by factors like marginal tax rates and firm risk.

The Pecking Order Theory elucidates how companies prioritize their financing options, primarily due to the information asymmetry between managers and investors. This information asymmetry leads companies to favor internal financing over external financing options [18].

When companies require external financing, they generally prefer debt issuance over equity issuance. This preference is driven by the aim to prevent undervaluation of equity, which could diminish the value of existing shareholders. Creditors, on the other hand, prioritize bankruptcy proceedings and are less concerned about the firm's valuation [19]. THence, companies opt for financing methods that entail minimal disclosure, commencing with retained earnings, followed by debt, and ultimately equity issuance. Empirical studies substantiate the Pecking Order Theory, indicating that profitable companies prioritize internal financing over external sources [20]. Profitable companies typically have substantial retained earnings and lower leverage. Furthermore, liquidity as a percentage of total assets exhibits a negative correlation with leverage, aligning with the theory that firms prioritize internal equity over debt [21]. The Pecking Order Theory can be summarized as emphasizing the influence of information advantages on companies' financial decisions, which leads them to prioritize internal financing, particularly retained earnings, over external sources such as debt and equity issuance [22].

3. METHODOLOGY AND RESULT OF THE RESEARCH

As of March 2024, there are 173 joint-stock companies registered in the MSE. For this study, we utilized a compilation of financial statements from a total of 2,417 samples of companies that submitted their financial statements between 2009 and 2022. However, due to insufficient financial data and missing information in the statements of some joint-stock companies within this sample, we excluded them from the analysis. Ultimately, we analyzed a total of 2,200 samples to ensure data accuracy and completeness. In our study, we investigated the relationship between the working capital ratio (ETA = Current assets/total assets) and the debt ratio (liabilities/total assets) with the return on assets (ROA = Profit before interest/total assets) and return on equity (net profit after tax/owners' equity). This analysis was conducted across five sectors and different company sizes categorized by the stock exchange. Essentially, we aimed to assess the optimal capital structure of companies using the working capital ratio and debt ratio, while evaluating financial performance through return on assets and return on equity.

[Table 1] Mean and median of indicators, 2009 to 2022

Year	Sample	ROA		ROE		ETA		DTA	
		Average	Median	Average	Median	Average	Median	Average	Median
2009	134	0.2%	0.2%	-3.2%	0.5%	38.8%	36.1%	41.3%	34.3%
2010	92	2.0%	1.1%	5.2%	2.8%	37.6%	34.0%	37.5%	30.8%
2011	181	0.9%	0.5%	0.2%	0.9%	39.3%	36.8%	44.5%	32.5%
2012	195	0.6%	0.4%	10.3%	0.8%	37.8%	34.9%	50.3%	33.9%
2013	194	-0.4%	0.1%	1.5%	0.4%	37.4%	30.5%	49.3%	33.5%
2014	189	-1.7%	0.0%	2.2%	0.0%	37.9%	31.6%	51.1%	38.0%
2015	118	-2.3%	0.0%	5.0%	0.2%	37.3%	31.0%	48.6%	35.3%
2016	176	-1.7%	0.0%	3.1%	0.2%	38.5%	29.5%	48.2%	33.1%
2017	164	-1.0%	0.0%	0.0%	0.5%	36.3%	28.0%	49.1%	31.3%
2018	166	0.0%	0.0%	0.6%	0.8%	38.0%	31.6%	56.2%	35.7%
2019	158	-0.4%	0.1%	5.4%	0.6%	39.7%	33.0%	52.4%	31.2%
2020	151	-0.9%	0.0%	7.3%	0.3%	37.7%	31.4%	50.0%	32.5%
2021	148	-0.2%	0.0%	3.5%	0.6%	40.9%	35.6%	51.0%	33.3%
2022	134	0.7%	0.0%	10.5%	1.1%	39.7%	30.1%	63.7%	38.0%
Дундаж		-0.35%	0.08%	3.61%	0.56%	38.31%	32.45%	49.80%	33.68%

Source. Researcher's estimation

The mean and median values of the financial ratios studied exhibit significant differences from 2009 to 2022 in show table 1. This variability in the normative level of each financial indicator is attributed to fluctuations in economic growth, market conditions, and numerous external and internal factors impacting the company, such as exchange rates, inflation, and loan interest rates. Additionally, notable discrepancies exist between the mean and median values of the ratios. For instance, the average return on equity (ROE) across the entire sample stands at 13.61 percent, while the median value is 0.56 percent. Notably, there are substantial differences observed in the years 2012, 2019, and 2022, indicating considerable variations in other indicators as well.

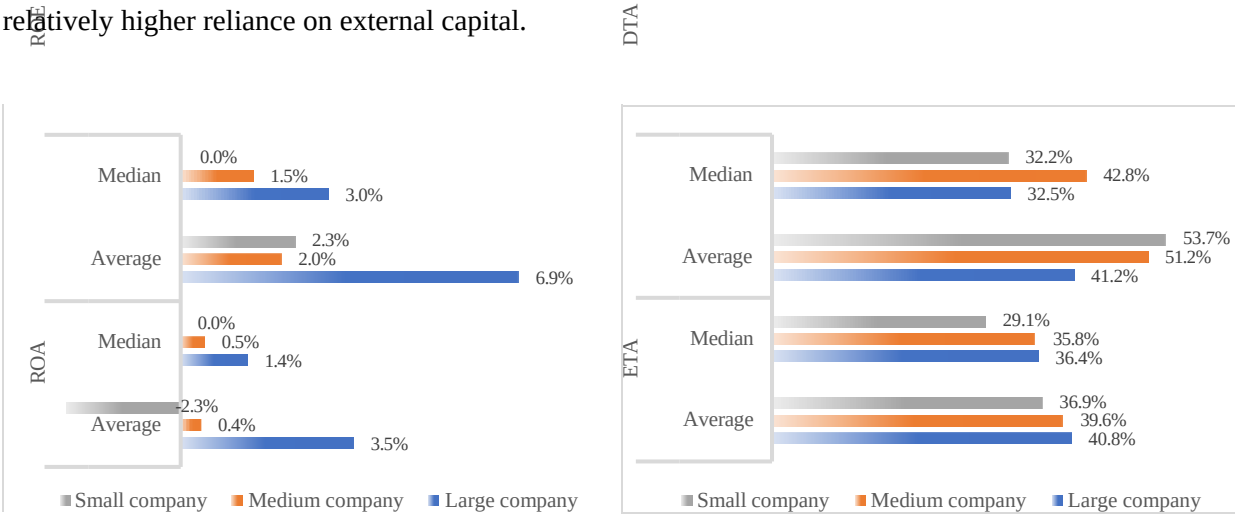
The MSE has categorized its registered joint-stock companies into five main sectors: processing (Sector A), manufacturing (Sector B), food and agriculture (Sector C), transportation (Sector D), and trading services (Sector E). Below are the mean and median values of the ratios for these sectors:

[Table 2] Industry mean and median for each indicator, 2009 to 2022

Sector	Sample	ROA		ROE		ETA		DTA	
		Average	Median	Average	Median	Average	Median	Average	Median
A	408	-0.6%	0.0%	8.4%	0.8%	38.9%	36.0%	59.0%	45.4%
B	494	2.3%	0.4%	5.0%	1.4%	43.1%	40.2%	51.0%	43.4%
C	309	-2.1%	0.0%	5.2%	0.5%	35.3%	29.0%	59.8%	45.4%
D	579	-0.7%	0.1%	1.9%	0.3%	34.2%	24.4%	36.4%	21.8%
E	410	-1.5%	0.0%	-1.6%	0.1%	40.0%	28.9%	50.6%	29.8%

Source. Researcher's estimation

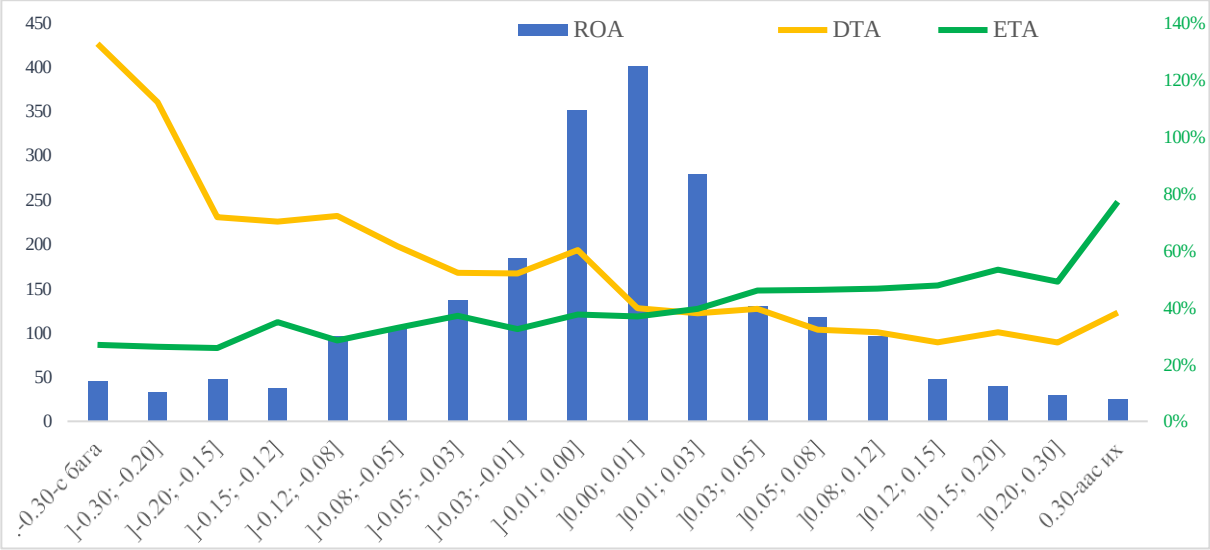
The mean and median values of the indicators also exhibit significant variations across sectors and even within each sector in table 2. For instance, in Industry A, the average return on equity (ROE) is 8.4 percent, while the median is 0.8 percent. This discrepancy indicates that while the average ROE for companies in this sector is 8.4 percent, half of the total sample has a return below 0.8 percent. The increase in the industry average is primarily attributed to the exceptional performance of a few companies in the sector with extremely high returns on equity. When ranked based on profitability, Sector B emerges as the most profitable, whereas Sector E ranks as the least profitable. In terms of working capital percentage, Sector D has the lowest figure at approximately 24%, whereas Sector B boasts the highest percentage at 40.2%. Sectors D and E exhibit the lowest debt ratios, with liabilities accounting for less than 30% of total assets, while Sectors A and C have relatively higher reliance on external capital.



[Figure 1] Mean and median for each indicator, by company size

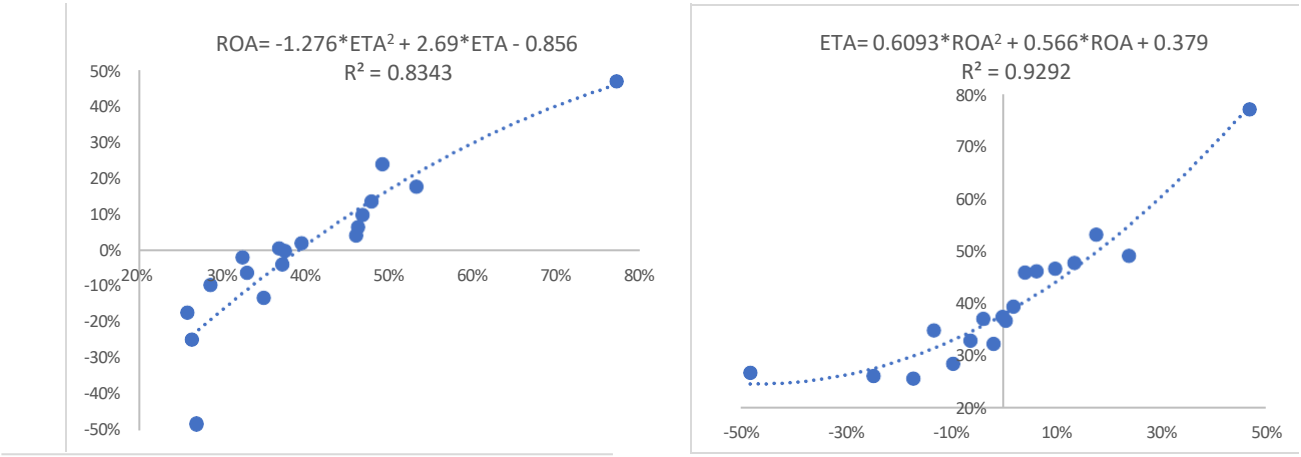
The graph presented above illustrates the average and median values of the indicators based on company size, as per the Minister of Finance's Order No. 2016.02. According to the Law on Non-profit Organizations, approved under Order No. 41 dated 3.4, enterprises

are classified as large if their total sales amount exceeds 1.5 billion MNT; otherwise, they are categorized as small or medium enterprises. However, with the enactment of the Law on Support of SMEs and Services in 2019, the classification criteria have been revised. Under this new law, enterprises with sales revenue up to 300 million are classified as micro, those with revenue between 300 million and 1 billion as small, those with revenue between 1 billion and 2.5 billion as medium, and those with revenue exceeding 2.5 billion as large. The average return on equity for large enterprises is 3percent, with a median of 6.9 percent. In contrast, small enterprises have an average return on equity of 2.3 percent, with a median of 0 percent. This indicates that the profitability of large enterprises surpasses that of small and medium enterprises. Additionally, as the size or sales income of an enterprise increases, the proportion of current assets in its portfolio also increases, while the percentage of liabilities in total assets decreases.



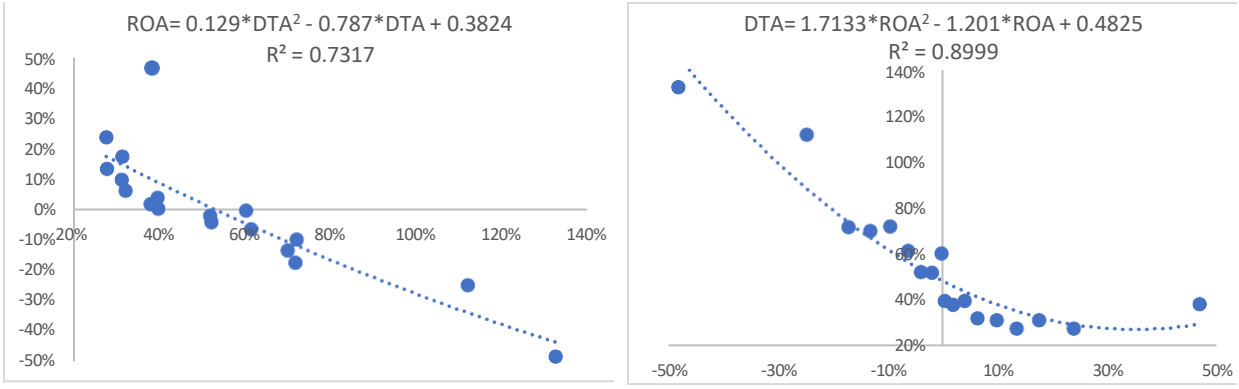
[Figure 2] Histogram of ROA and ETA, DTA correlation

The relationship observed in the study indicates that as the ETA (working capital ratio) of surveyed public companies increases, so does the ROA (return on assets). Conversely, a lower DTA (debt ratio) is associated with a higher ROA. Specifically, a higher working capital to total assets ratio or a lower liabilities to total assets ratio corresponds to a higher return on equity. However, it's important to note that this relationship is non-linear and follows a quadratic function rather than a linear one.



[Figure 3] Nonlinear relationship between ROA and ETA

The coefficient of determination for the non-linear relationship between Return on Assets (ROA) and the Working Capital Ratio (ETA) is 83.4%, whereas it is 73.2% for the non-linear relationship with the Debt Ratio (DTA). Conversely, the coefficient of determination for the non-linear relationship between ROA and ETA is 92.9%, and 89.9% for DTA.



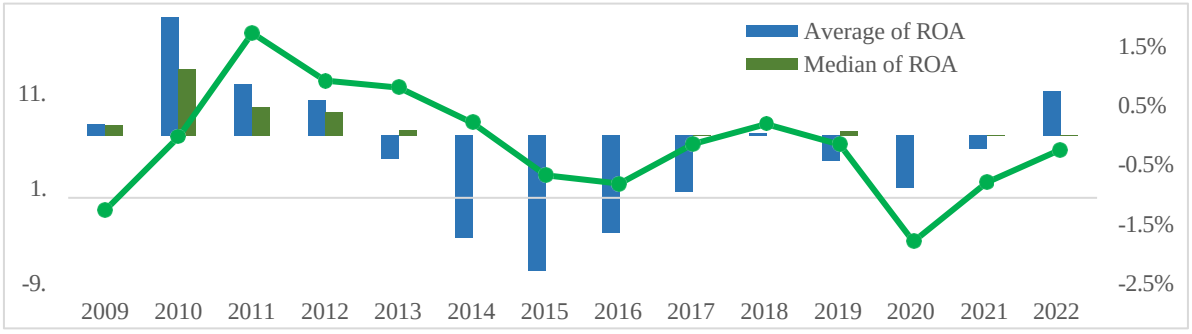
[Figure 4] Nonlinear relationship between ROA and DTA

The correlation between Return on Assets (ROA) and the Working Capital Ratio (ETA) based on company size was 57.6 percent, and -42.8 percent for the correlation between ROA and the Debt Ratio (DTA) in table 3. It was observed that as the size (annual sales revenue) of the company increased, the Return on Total Assets also increased. It includes:

[Table 3] Mean and median of financial ratios by company size

Company size	ROA		ROE		ETA		DTA	
	Average	Median	Average	Median	Average	Median	Average	Median
Large company	3.5%	1.4%	6.9%	3.0%	40.8%	36.4%	41.2%	32.5%
Medium company	0.4%	0.5%	2.0%	1.5%	39.6%	35.8%	51.2%	42.8%
Small company	-2.3%	0.0%	2.3%	0.0%	36.9%	29.1%	53.7%	32.2%

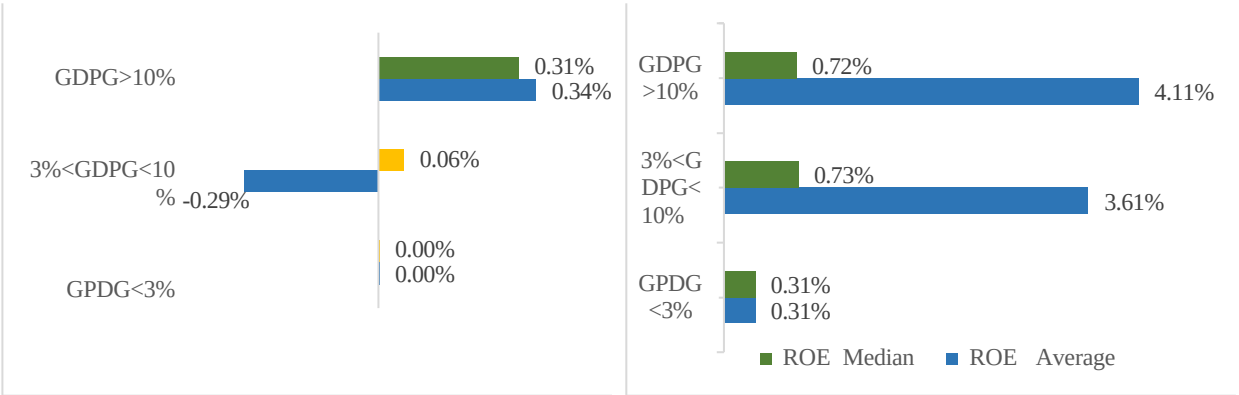
A positive and robust correlation is evident between real GDP or economic growth and the total current assets of public companies. This indicates that economic growth tends to be higher during years when the average and median return on capital of surveyed joint-stock companies is also high. It includes:



[Figure 5] Economic growth and return on capital (average and median)

In years characterized by GDP growth (GDPG) or economic growth below 3% (2009, 2015, 2016, 2020, 2021), the mean and median Return on Equity (ROE) is 0.31%. Conversely, during years when GDPG ranges between 3% and 10% (2010, 2014, 2017-2019, 2022), the average ROE is 3.61% with a median of 0.73%. In contrast, in years with GDPG exceeding 10% (2011-2013), the average ROE stands at 4.11% with a median of 0.31%. It includes:

[Figure 6] Normative level of profitability depending on economic growth



Economic growth serves as a primary driver for the rise in profits and income of companies. However, it's worth noting that the increase in profits and income of companies can also contribute to economic growth. In this context, the relationship between economic growth and the profitability of companies listed in the BSE was assessed using Granger's test [23] with a lag order set to 1. It includes:

[Table 4] Granger causality test results (lags-1)

Pairwise Granger Causality Tests	Sampe: 2009-2022			
	Null Hypothesis:	Obs	F-Statistic	Prob.
ROE ба GDPG	GDPG does not Granger Cause ROA	13	1.10060	0.3188
	ROA does not Granger Cause GDPG		11.5782	0.0067
	Null Hypothesis:	Obs	F-Statistic	Prob.
ROE ба GDPG	GDPG does not Granger Cause ROE	13	2.23902	0.1654
	ROE does not Granger Cause GDPG		9.14651	0.0128

The Granger test results indicate that Gross Domestic Product Growth (GDPG) table 4 is not Granger causal to Return on Assets (ROA) and Return on Equity (ROE). However, ROA and ROE are Granger causal to GDPG at a significance level exceeding 95%. This implies that the profitability indicators of companies are not dependent on economic growth, but rather, economic growth is influenced by the profitability indicators of companies. Finally, we evaluated the effects of ROA and ROE on economic growth using the Least Squares method, and the estimation results were found to be statistically significant. It includes:

$$\text{GDPG} = 3.71208955224 + 13.4053482587*\text{ROA}(-1) \qquad R^2 = 0.598872$$
$$\text{t-stat} \qquad 3.121617*** \qquad 4.052493***$$

$$\text{GDPG} = 2.47714930925 + 5.60779755579*\text{ROE}(-1) \qquad R^2 = 490633$$
$$\text{t-stat} \qquad 1.530099 \qquad 3.255060***$$

4. CONCLUSION

In this study, our objective was to ascertain the principal financial ratios of joint-stock companies registered in the MSE, specifically focusing on the percentage of working capital and the normative level of the debt ratio, alongside examining their correlation with profitability indicators. Our findings led to the following conclusions. It includes:

Given the significant disparity between the average and median values of financial ratio indicators among joint-stock companies, it is advisable to utilize the median value instead

of the average value when determining financial ratios and the normative level of the industry. This approach will yield a more realistic assessment and conclusion.

The normative values of financial ratio indicators for companies fluctuate annually, influenced by industry characteristics and company size. Given their dependence on numerous environmental factors, it is suitable to establish the normative level of financial ratios for each economic cycle, industry, and company size.

The normative values of financial ratio indicators for companies fluctuate annually, influenced by industry characteristics and company size. Given their dependence on numerous environmental factors, it is suitable to establish the normative level of financial ratios for each economic cycle, industry, and company size.

As the size of the company increases, there is an increase in both the percentage of profitability and working capital, accompanied by a decrease in the debt ratio. For instance, if the debt ratio of large companies is 41.2%, medium-sized companies are at 51.2%, and small companies are at 53.7%, it can be inferred that the normative level is being met.

The profitability indicators of companies exhibit a positive non-linear relationship with the percentage of working capital and a negative non-linear relationship with the debt ratio. It is observed that profitability decreases up to a certain level of turnover ratio and debt ratio, but it begins to increase again beyond that level.

In years characterized by high economic growth, the average and median profitability indicators of joint-stock companies also exhibit elevated levels. However, economic growth itself does not directly cause an increase in the profitability of companies; instead, there is a notable correlation where economic growth experiences a significant change one year after the profitability of companies increases. For instance, if the median value of return on assets (ROA) for companies rises by 0.1 points, economic growth sees an increase of 1.34 percent after one year. Moreover, when economic growth is below 3%, the average Return on Equity (ROE) stands at 4.11%; within the range of 3 to 10 percent economic growth, the average ROE is 3.61%; and for economic growth exceeding 10 percent, the average ROE rises to 4.11%.

To enhance this study, it is recommended to establish the normative level of other financial ratios not initially included. Additionally, investigating the relationship between inflation rates, interest rates, and other macroeconomic indicators besides economic growth would provide valuable insights.

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APPENDIX

Appendix 1. The relationship between economic growth and ROA

Dependent Variable: GDPG
Method: Least Squares
Date: 03/19/24 Time: 14:38
Sample (adjusted): 2010 2022
Included observations: 13 after adjustments

Variable	Coefficient	Std. Error	t-Statistic	Prob.
C	3.712090	1.189156	3.121617	0.0097
ROA (-1)	13.40535	3.307926	4.052493	0.0019

R-squared	0.598872	Mean dependent var	6.186923
Adjusted R-squared	0.562406	S.D. dependent var	5.561496
S.E. of regression	3.678978	Akaike info criterion	5.583785
Sum squared resid	148.8836	Schwarz criterion	5.670700
Log likelihood	-34.29460	Hannan-Quinn critter.	5.565920
F-statistic	16.42270	Durbin-Watson stat	1.731750
Prob(F-statistic)	0.001907		

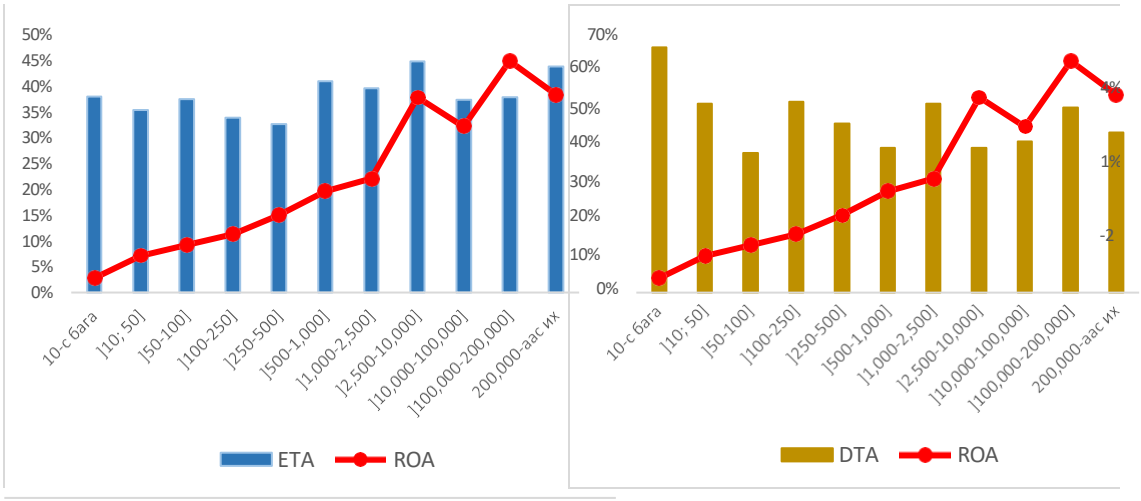
Appendix 2. The relationship between economic growth and ROA

Dependent Variable: GDPG
Method: Least Squares
Date: 03/19/24 Time: 14:38
Sample (adjusted): 2010 2022
Included observations: 13 after adjustments

Variable	Coefficient	Std. Error	t-Statistic	Prob.
C	2.477149	1.618947	1.530099	0.1542
ROE (-1)	5.607798	1.722794	3.255060	0.0077

R-squared	0.490633	Mean dependent var	6.186923
Adjusted R-squared	0.444326	S.D. dependent var	5.561496
S.E. of regression	4.145735	Akaike info criterion	5.822675
Sum squared resid	189.0583	Schwarz criterion	5.909591
Log likelihood	-35.84739	Hannan-Quinn critter.	5.804810
F-statistic	10.59541	Durbin-Watson stat	1.232872
Prob(F-statistic)	0.007666		

Appendix 3. Average ROA by company size



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The impact of entrepreneur's age on serial entrepreneurship speed: the moderating effect of positive gains after failure

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The impact of entrepreneur's age on serial entrepreneurship speed: the moderating effect of positive gains after failure

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Abstract— This study analyzes the impact of entrepreneurs' age and positive outcomes after failure on the speed of serial entrepreneurship. Generally, older entrepreneurs tend to pursue serial ventures more rapidly; however, a clear explanation for this phenomenon is still lacking. This research identifies age and positive gains after failure as key variables and empirically analyzes their effects on the speed of serial entrepreneurship, drawing on prior studies. The results indicate that age alone does not lead to faster serial entrepreneurship. Instead, older entrepreneurs who experience higher positive gains after failure are more likely to re-enter the entrepreneurial space quickly. This suggests an interaction between entrepreneurs' age and positive gains after failure that positively influences entrepreneurial success, underscoring the need for tailored training programs for entrepreneurs.

Keywords—Serial entrepreneurship, Entrepreneur`s age, Serial entrepreneurship speed, Positive gains after failure

1. INTRODUCTION

It has been pointed out that long-term support measures are needed to activate the domestic serial entrepreneurship ecosystem and improve the survival rates of businesses. According to a report published by the Small and Medium Business Administration on October 26, 2023, two out of three newly established businesses in South Korea fail within five years, which is 11.6 percentage points higher than the OECD average of 54.6%. Consequently, the report emphasizes

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the need to strengthen the support system for serial entrepreneurship to enhance the survival rate of domestic startups. According to McGrath [1], entrepreneurship is a process fraught with uncertainty and risk, which can lead to anxiety for entrepreneurs. However, it can also be argued that entrepreneurs can leverage failure as a valuable learning experience. Through failure, they can identify their mistakes and better prepare for future success. The entrepreneurial ecosystem should allow for failure and promote learning throughout this process. Serial entrepreneurship signifies the pursuit of new challenges and opportunities, and research indicates that serial entrepreneurs tend to start their initial businesses at a younger age compared to other entrepreneurs. However, there is a lack of research on the relationship between the characteristics and behaviors of serial entrepreneurs, presenting new opportunities for investigation.

The main objectives of this study are as follows: First, to thoroughly review prior research related to the age of entrepreneurs, the speed of serial entrepreneurship, and positive gains after failure, and to establish a research model and hypotheses based on this review. Second, to verify the impact of entrepreneurs' age on the speed of serial entrepreneurship and examine the differences in the effects of positive gains after failure on the relationship between entrepreneurs' age and the speed of serial entrepreneurship. Third, to provide useful implications for entrepreneurs and practitioners in the field of entrepreneurship based on the analytical results obtained.

2. THEORETICAL BACKGROUND

2.1. THE CONCEPT OF SERIAL ENTREPRENEURSHIP

According to Ucbasaran et al.[2], serial entrepreneurship plays a decisive role in entrepreneurial intent and often requires significant investments of time, money, and effort. Additionally, Yoon Nam-soo [3] emphasizes the importance of serial entrepreneurship, stating that strong entrepreneurial intention is essential in this process. This suggests that serial entrepreneurship is not merely about giving up after a single failure; rather, it involves embarking on new challenges based on the lessons learned from those failures to achieve success.

Serial entrepreneurship is distinct from portfolio entrepreneurship. As posited by Westhead and Wright [4], it is founded on the idea that some entrepreneurs repeatedly start businesses and achieve success. Baron and Ensley [5] explain that this characteristic enables serial entrepreneurs to continuously improve their skills and strategies, providing opportunities for better outcomes in subsequent ventures.

Serial entrepreneurs act as agents who facilitate and build new ventures, playing a crucial role in driving innovation and growth in both the economy and society. They take on various risks while simultaneously becoming significant beneficiaries. Through this process, serial entrepreneurs optimize diverse production means and establish viable re-systems. The serial entrepreneurship process is a vital economic activity that significantly impacts economic growth and development. By actively utilizing innovative mechanisms, serial entrepreneurs combine various production means and provide new products or services to the market, thereby creating economic value. This role promotes economic activity, generates new jobs, and triggers changes

in existing industries. Thus, serial entrepreneurs serve as essential agents in the economy and society by merging diverse production means through innovative approaches and offering a variety of goods and services [6].

Parker [7] concluded from his investigation of long-term entrepreneurial activities that successful transitions between ventures generate profits, providing insight into the positive impact of serial entrepreneurship on performance. Furthermore, Sarasvathy et al. [8] argue that failed businesses offer important learning opportunities for serial entrepreneurs, suggesting that the lessons learned from failures can be as significant as those from successful ventures.

2.2. ENTREPRENEUR`S AGE

In research on entrepreneurship, age is closely related to entrepreneurial intent, motivation, and behavior [9]. Specifically, the age of serial entrepreneurs is associated with favorable attitudes toward serial entrepreneurship. Studies indicate that as age increases, so do tendencies toward risk aversion [10]. This shift in priorities can affect an entrepreneur's willingness to create economic or non-economic social value, depending on their life stage.

Younger individuals are more likely to transition to wage employment after a startup failure, as they still have available job opportunities [11]. Consequently, the inclination toward serial entrepreneurship among younger age groups tends to be relatively low. In contrast, middle-aged individuals are more likely to turn failures into opportunities for serial entrepreneurship. Despite facing challenges related to significant career changes, they often proactively seek new challenges, drawing on their accumulated experience and expertise. Such experiences can be critical for finding solutions through trial and error [12]. Thus, higher rates of serial entrepreneurship after failure are expected in this age group.

Research by Jang Young-mi [13] suggests that failures in necessity-driven entrepreneurship can negatively impact older entrepreneurs' likelihood of pursuing serial ventures. For older individuals, these ventures are often highly competitive, and insufficient preparation increases the risk of failure. Additionally, securing funding for serial entrepreneurship can be challenging post-failure, and older entrepreneurs may exhibit a diminished willingness to learn new skills, viewing serial entrepreneurship as a greater risk. Lee Jong-seon [14] also noted that older failed entrepreneurs tend to show lower tendencies toward serial entrepreneurship, indicating inconsistencies in research findings regarding age and serial entrepreneurship.

The age of entrepreneurs is a critical variable in understanding how their behaviors, attitudes, and business intentions are influenced. Age encompasses not only a numerical value but also diverse experiences, skills, and perceptions that evolve over time [15]. Older entrepreneurs often possess rich experiences and resources that aid in decision-making and problem-solving. Conversely, younger entrepreneurs may demonstrate higher risk tolerance and adaptability to technological changes, fostering the development of innovative ideas and business models [16].

In summary, the age of entrepreneurs signifies more than just a number; it is an important variable influencing business activities through their experiences and characteristics. This study categorizes entrepreneurs into five distinct age groups for a more detailed examination: under 18, 18-29, 30-44, 45-60, and over 60. This classification aims to enhance understanding of the unique challenges faced by entrepreneurs at different life stages, allowing for more accurate assessments of how age impacts business behavior and performance.

2.3. SERIAL ENTREPRENEURSHIP SPEED

The serial entrepreneurship speed refers to the time it takes for an entrepreneur to exit a previously established business or to recover from failure and start a new venture. This process often involves identifying new opportunities based on prior experiences and embarking on new challenges. The serial entrepreneurship speed includes not only addressing previous failures but also the time taken after exiting (selling or transferring) the prior business or terminating it in another manner. This phase encompasses discovering new opportunities or experimenting with different business models.

Previous research has confirmed that serial entrepreneurship speed is related to entrepreneurial intent, indicating that higher entrepreneurial intent correlates with faster serial entrepreneurship [17]. This suggests that an entrepreneur's desire to quickly pursue new opportunities is informed by prior experiences.

Many researchers consider new entrepreneurial activities to be socially and economically significant [18] and have shown considerable interest in serial entrepreneurship as a key factor in entrepreneurial activities [19]. Serial entrepreneurship speed is closely linked to entrepreneurial intent, with higher levels of intent leading to faster entrepreneurial pursuits [17]. Burton et al. [20] emphasized the importance of understanding the causes and consequences of previous failure experiences to analyze the trajectories of entrepreneurs in new economies. However, research specifically on serial entrepreneurship speed remains limited, with existing studies primarily focusing on the speed of initial startups or the time required to create new ventures [21].

According to human capital theory [22], individuals with extensive prior entrepreneurial experience are better equipped to execute new ventures more swiftly. Prior entrepreneurial experience positively influences future entrepreneurial behaviors and the speed of new venture creation [23]. This experience equips entrepreneurs with applicable knowledge, enabling them to gather and process information more efficiently, thereby enhancing the speed of new ventures [21].

2.4. POSITIVE GAINS AFTER FAILURE

Entrepreneurial failure can be interpreted in various ways and has been analyzed from different perspectives by researchers such as Shepherd and Haynie [24]. Ucbasaran et al. [25] define entrepreneurial failure as the cessation of business operations when the entrepreneur fails to meet the minimum economic viability they had established. Amankwah-Amoah et al. [26] propose four distinct stages that entrepreneurs typically go through after experiencing failure: 1.Grief and Despair Stage: In this initial stage, entrepreneurs confront the sorrow and despair resulting from failure. The shock of the experience leads to self-doubt and a significant loss of confidence. 2.Transition Stage: Here, entrepreneurs begin to accept their failure and seek to overcome it, moving toward a new beginning. This involves reflecting on past experiences and learning from them. 3.Formation Stage: In this stage, entrepreneurs explore new opportunities and develop new business models, preparing to establish and realize a new vision. 4.Legacy Stage: The final stage involves successfully launching and stabilizing a new venture. Entrepreneurs focus on achieving

success and building their new business.

These four stages illustrate how entrepreneurs can overcome failure and seek new opportunities, providing essential guidance for successful serial entrepreneurship. The authors conclude that experiences of failure can influence subsequent entrepreneurial behaviors. However, while hypotheses have been suggested regarding how failure experiences connect to entrepreneurs' ability to start and grow new ventures [25], there remains a lack of clear explanations about how the lessons learned from failure mediate the relationship between failure experiences and new venture outcomes.

Failure can serve as a valuable learning opportunity for entrepreneurs with failed businesses [1], but it can also be an emotionally and psychologically challenging experience that hinders learning [27]. Even when learning occurs, entrepreneurs must effectively share and disseminate the new knowledge gained from their failures. Through this sharing, they can leverage lessons learned to discover new opportunities and successfully engage in serial entrepreneurship [28]. However, this learning process can come at a high cost, resulting in increased psychological, social, and financial burdens for entrepreneurs. It involves not just accumulating knowledge but actively using it to identify and realize new opportunities, playing a crucial role in the successful serial entrepreneurship and ongoing competitive advantage of entrepreneurs.

Corbett [29] presents a significant perspective on learning from failure, arguing that it is not merely about knowledge accumulation; rather, it involves developing the cognitive ability to utilize past failure experiences effectively. Entrepreneurs can develop new perspectives and approaches based on insights gained from failure, applying these to future opportunities to achieve success. This view emphasizes the growth and development entrepreneurs undergo through learning from failure, suggesting that failure should not be viewed solely as a negative experience but rather as an opportunity for learning and growth. This aligns with theoretical frameworks that recognize learning as a capacity for change and development. Thus, learning from failure becomes an essential component in enhancing entrepreneurs' abilities to recognize and exploit new opportunities.

3. RESEARCH MODEL AND HYPOTHESIS

3.1. RESEARCH MODEL

In this study, the entrepreneur's age is established as the independent variable, while positive gains after failure are treated as the moderating variable. The serial entrepreneurship speed is selected as the dependent variable. To test the hypotheses, regression analysis and moderated moderation effect analysis will be conducted. The research model for this study is as follows:

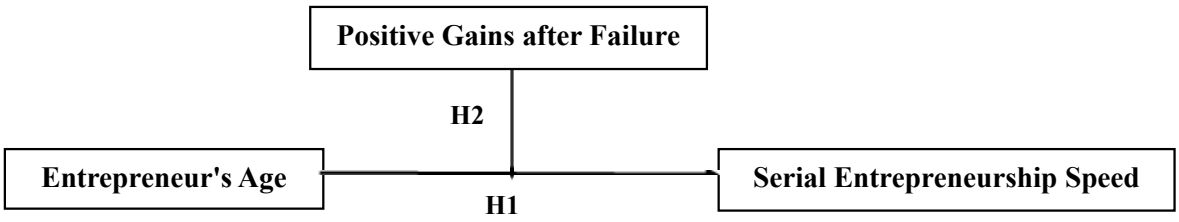

Fig. 1 Research Model

3.2. HYPOTHESIS

Based on the research model, this study aims to establish the following hypotheses for testing. First, we will examine the relationship between the entrepreneur's age and serial entrepreneurship speed. Second, we will investigate the moderating effect of positive gains after failure on the relationship between the entrepreneur's age and serial entrepreneurship speed. In total, two hypotheses have been formulated. The background for establishing these hypotheses and the specific hypotheses are as follows:

3.2.1 ENTREPRENEUR'S AGE AND SERIAL ENTREPRENEURSHIP SPEED

This study aims to analyze the impact of entrepreneurs' experiences and age on the serial entrepreneurship process. Research indicates that as age increases, individuals tend to make fewer significant career changes [30]. Younger individuals often lack the development of professional networks and relevant knowledge, leading to lower costs associated with transitioning to new careers [30]. Consequently, even if they do not choose serial entrepreneurship, they face less financial burden and can more easily begin new careers through employment after a startup failure.

In contrast, studies suggest that middle-aged and older individuals find it increasingly difficult to gain employment as wage workers as they age. Younger individuals, who often lack social experience, may choose employment to build social skills and better prepare for future entrepreneurial endeavors. Therefore, it is expected that younger entrepreneurs will have a lower tendency toward serial entrepreneurship after a startup failure.

For middle-aged individuals, the tendency for startup failure to lead to serial entrepreneurship may differ. Middle-aged entrepreneurs may face constraints in choosing broader professional categories, and given their age and potential career interruptions, they might struggle to find satisfying job opportunities [31]. However, middle-aged individuals can actively engage in new challenges, and past failure experiences may help them identify better solutions through trial and error [32]. They may gain confidence in their ability to learn from failures and improve past decisions, leading to greater interest in serial entrepreneurship [33].

For older entrepreneurs, serial entrepreneurship may be more challenging post-failure. Older individuals often require a steady income, but barriers to re-employment increase, suggesting that the higher the age of failed entrepreneurs, the more negative the impact on serial entrepreneurship. This trend is particularly evident in countries lacking economic stability, where many older individuals tend to choose subsistence entrepreneurship [34]. Additionally, as age increases, the willingness to take on additional risks may decrease, along with the desire to learn new things, resulting in lower potential for serial entrepreneurship [35].

Therefore, preferences for entrepreneurship may decline with age, leading individuals to feel they lack sufficient time and energy to seize business opportunities or pursue serial entrepreneurship [17].

Hypothesis 1: As the entrepreneur's age increases, the speed of serial entrepreneurship will decrease.

3.2.2 POSITIVE GAINS AFTER FAILURE AND SERIAL ENTREPRENEURSHIP SPEED

According to Shepherd [27], failure is an important opportunity for entrepreneurs to gain valuable lessons. Cope [36] emphasizes that learning from failure can take on abstract and generalized forms, enabling entrepreneurs to respond effectively to challenges in new business situations. Sarasvathy and Menon [37] argue that experiences gained through failure serve as essential tools for understanding "what works and what doesn't." Politis [38] describes failure as a "stepping stone" for learning to find new opportunities and improve business processes. Entrepreneurs derive abstract and generalized learning from their failure experiences, which can help develop cognitive early warning systems and facilitate corrective actions in subsequent ventures [39]. These perspectives underscore the importance of continuous learning and growth from failure. Positive gains after failure can assist in quickly identifying new opportunities and taking appropriate actions. Effectively applying these positive gains can enhance the response speed to issues arising in serial entrepreneurship. Thus, it is expected that a high level of positive gains after failure will lead to faster serial entrepreneurship speed for the entrepreneur.

Depending on the entrepreneur's age, recovery from failure and the acquisition of high-level learning capabilities are considered part of a unique learning process [40]. Research indicates that older entrepreneurs tend to possess rich experiences that facilitate deeper learning. They are expected to gain more lessons from failure and be more skilled at applying these lessons to new opportunities. In this regard, Hajizadeh and Zali [41] found that the ability of older entrepreneurs to learn from past business experiences plays a critical role in successfully starting and growing new businesses. Consequently, it is anticipated that older entrepreneurs will have a relatively higher capacity to connect their experiences of business failure with the outcomes of new ventures through learning from failure, advancing existing research on business failures. This underscores the notion that experiences of startup failures and subsequent learning processes can play a decisive role in the success of new businesses.

Hypothesis 2: Positive gains after failure will strengthen the relationship between the entrepreneur's age and serial entrepreneurship speed. Among individuals of the same age, those with high positive gains after failure will exhibit faster serial entrepreneurship speed than those with lower gains.

3.3. VARIABLE MEASUREMENT

The operational definitions of the variables used in the empirical analysis are presented in Table 1.

Table 1. Survey sample description

Variable Type	Measurement Variable	Operational Definition	Source
Dependent Variable	Serial Entrepreneurship Speed	How long did it take to start a new venture after the previous failure? (1=Over 2 years, 2=1-2 years, 3=6 months-1 year, 4=Under 6 months)	Kautonen et al., 2015
Independent Variable	Entrepreneur's Age	Age group (1=Under 18, 2=18-29, 3=30-44, 4=45-60, 5=Over 60)	Baù et al., 2017
Moderator Variable	Positive Gains After Failure	● Failure in entrepreneurship provided valuable lessons that could not be learned otherwise. ● The experience of failure helped build resilience and perseverance. ● I gained insights into areas like financial management, market research, and leadership.	Cope, 2005
Control Variables	Economic Family Support	● I received financial support from my family.	Rau, 2014
	Psychological Family Support	● My family provides the moral support necessary for my business success. ● I receive the emotional help and support I need from my family.	
	Self-Efficacy	● How confident are you in successfully identifying new business opportunities? ● How confident are you in successfully creating new products? ● How confident are you in thinking creatively? ● How confident are you in successfully commercializing ideas or new developments?	Zhao et al., 2005
	Causes of Failure	● After my entrepreneurial failure, I thought my personality lacked motivation for entrepreneurship. ● I thought my personality was unsuitable for entrepreneurship after my failure.	Piadehbasm and Higgins, 2016
	Operating Period	How long have you officially operated your current business? (1=Less than 1 year, 2=1-2 years, 3=2-3 years, 4=Over 3 years)	

3.4. ANALYSIS METHOD

In accordance with the objectives of this study, we aimed to: 1) examine whether the entrepreneur's age affects serial entrepreneurship speed, and 2) verify the moderating effect of positive gains after failure on the relationship between the entrepreneur's age and serial entrepreneurship speed.

The survey data collected for this study were coded and analyzed using SPSS 26.0. To assess the moderating effect of positive gains after failure in the relationship between the entrepreneur's age and serial entrepreneurship speed, we utilized Model 1 of the PROCESS macro 4.1. In this analysis, the entrepreneur's age was treated as the independent variable, serial entrepreneurship speed as the dependent variable, and positive gains after failure as the moderator. Control variables included economic family support, psychological family support, self-efficacy, causes of failure, and operating period.

4. ANALYSIS OF RESEARCH RESULTS

4.1 DATA COLLECTION AND RESEARCH SAMPLE

A survey was conducted among entrepreneurs who have experienced business failure and are currently engaged in serial entrepreneurship. The survey included a total of 109 participants, all of whom are current entrepreneurs from various regions in the United States. The data collection took place over one week in September 2023 using the Survey Monkey platform.

Table 2. Demographic Characteristics

Category		Freq	%	Category		Freq	%
Gender	Male	68	62.4%	Experience	Yes	109	100%
	Female	41	37.6%		No	0	0.0%
Entrepreneur's Age	18-29 years	24	22.0%	Current Startup Duration	Less than 1 year	13	11.0%
	30-44 years	38	34.9%		1~2 years	34	31.2%
	45-60 years	39	35.8%		2~3 years	32	29.4%
	60 years and older	8	7.3%		More than 3 years	31	28.4%
Entrepreneurship Experience Level	2nd time	61	56.0%	Education	High School	11	9.2%
					Technical	7	6.4%
	3rd time	31	28.4%		College	14	12.8%
	4th time	11	10.1%		Bachelor	37	33.9%
	5 or more	6	5.5%		Master	31	28.4%
					Doctorate	10	9.2%

4.2 VARIABLE MEASUREMENT

4.2.1. DESCRIPTIVE STATISTICS

The results obtained from the sample data indicate that, in the analysis of serial entrepreneurship speed among the 109 respondents, the dependent variable—serial entrepreneurship speed-has a mean of 2.513 (standard deviation SD = 1.005). This suggests that, on average, it takes about one year for re-entrepreneurship. The independent variable, entrepreneur's age, has a mean of 3.284 (SD = 0.893), indicating a tendency for entrepreneurs to be predominantly over the age of 40. Further examination of the correlation between serial entrepreneurship speed and age would be insightful. The moderating variable, positive gains after failure, has a mean of 5.473 (SD = 1.386), suggesting that respondents generally experience positive outcomes after failure during their re-entrepreneurial efforts.

Table 3. Descriptive Statistics

Variable		N	Mean	S.D.	Skewness	Kurtosis
Dependent	Serial Entrepreneurship Speed	109	2.513	1.005	-.011	-1.059
Independent	Entrepreneur's Age	109	3.284	.893	.039	-.855
Moderator	Positive Gains After Failure	109	5.473	1.386	-1.067	.917
Control Variable	Economic Family Support	109	3.858	2.405	.040	-1.588
	Psychological Family Support	109	5.663	1.281	-.788	-.103
	Self-efficacy	109	5.420	1.294	-.569	-.586
	Cause of Failure	109	4.823	1.518	-.155	-.537
	Duration of Operation	109	2.752	.992	-.177	-1.072

4.2.2. RELIABILITY ANALYSIS AND EXPLORATORY FACTOR ANALYSIS

This study utilized Cronbach's Alpha coefficient to estimate the internal consistency among the items. The results indicated that the reliability coefficients for all measured variables were above 0.5, suggesting high internal consistency among the items. For the three items related to positive gains after failure in the context of serial entrepreneurship speed, the Cronbach's Alpha value was found to be .845, indicating that this variable serves as a stable and reliable measurement tool.

Since the entrepreneur's age and speed of serial entrepreneurship were measured on a nominal scale, no reliability analysis was conducted for these variables. This means that the internal consistency or stability of the measurement tools was not evaluated; however, it suggests that each variable meets the validity assessment criteria and adequately reflects their operational definitions as measurement tools.

The eigenvalues indicate the importance of each variable concerning the factors identified in the exploratory factor analysis. As shown in Table 4, the pattern and distribution of all factor loadings were found to be appropriate.

Table 4. Exploratory Factor Analysis

	1	Commonality Values
Positive Gains After Failure 1	.900	.810
Positive Gains After Failure 2	.847	.718
Positive Gains After Failure 3	.895	.802
Total	2.329	
% Variance	77.629	
Cumulative %	77.629	
KMO (Kaiser-Meyer-Olkin) Sample Adequacy: .720		
Bartlett's Test of Sphericity: $\chi^2 = 147.251$ (df = 3, $p < .000$)		

Extraction Method: Principal Axis Factoring
Rotation Method: Varimax with Kaiser Normalization

4.2.3. CORRELATION ANALYSIS

In this study, Pearson's correlation analysis was employed to examine the relationships between the main variables-entrepreneur's age, positive gains after failure, and serial entrepreneurship speed-while controlling for economic family support, psychological family support, self-efficacy, causes of failure, and operational duration. The results of the correlation analysis are presented in Table 5.

Table 5. Correlation Analysis

Variable	1	2	3	4	5	6	7	8
Serial Entrepreneurship Speed	1							
Entrepreneur's Age	·073	1						
Positive Gains After Failure	-·057	.239*	1					
Economic Family Support	-.226*	-.030	·132	1				
Psychological Family Support	-.177	.248**	.554**	·099	1			
Self-efficacy	.005	.129	.594**	.254**	·512**	1		
Cause of Failure	-.257*	.045	.323**	.613**	.443**	.446**	1	
Duration of Operation	-.196*	·132	.259**	-.042	-·012	.116	-.115	1

*$p<.05$, **$p<.01$

4.2.4. REGRESSION ANALYSIS

To verify the relationship between the entrepreneur's age, positive gains after failure, and serial entrepreneurship speed, the model summary and coefficients are presented in Table 6. Model 1 includes only the control variables related to the entrepreneur's background. Model 2 incorporates these control variables along with the independent variables: the entrepreneur's age and positive gains after failure. Model 3 aims to analyze the moderating effect of positive gains after failure on the relationship between the entrepreneur's age and serial entrepreneurship speed by additionally including the interaction term "entrepreneur's age * positive gains after failure." Examining the explanatory power of each regression model, Model 1 (R^2 = .183, F = 4.617), Model 2 (R^2 = .205, F = 3.717), and Model 3 (R^2 = .237, F = 3.891) all demonstrate a good fit. The Durbin-Watson statistic for testing the independence of residuals is 2.077, indicating independence as it is close to 2. Based on Model 3, the results of hypothesis testing reveal that the effect of the entrepreneur's age on serial entrepreneurship speed is significant, thus supporting Hypothesis 1 (β = .335, t = 2.506). Additionally, Hypothesis 2 is also supported, as the moderating effect of positive gains after failure between the entrepreneur's age and serial entrepreneurship speed is significant (β = -.755, t = -2.066).

Table 6. Regression Analysis Results

		Model 1		Model 2		Model 3	
		β	t	β	t	β	t
Constant			7.648		5.931		4.807
Control Variable	Economic Family Support	-.147	-1.261	-.143	-1.233	-.149	-1.302
	Psychological Family Support	-.209	-1.873	-.275	-2.270	-.260	-2.173
	Self-efficacy	.284	2.572	.252	2.108	.223	1.879
	Cause of Failure	-.231	-1.742	-.227	-1.718	-.240	-1.842
	Duration of Operation	-.264	-2.894	-.300	-3.145	-.323	-3.409
Direct Effects	Entrepreneur's Age (A)			.134	1.440	.335	2.506
	Positive Gains After Failure (B)			.084	676	.761	2.175
Moderating Effect	A × B					-.755	-2.066
F		4.617		3.717		3.891	
R^2		.183		.205		.237	
Adjusted R^2		.143		.150		.176	
Change in R^2		.183		.022		.033	

Dependent Variable: Serial Entrepreneurship Speed

4.2.5. PROCESS MACRO

In this study, Hayes' PROCESS macro Model 1 was utilized to analyze the moderating effect of positive gains after failure on the relationship between the entrepreneur's age and serial entrepreneurship speed. Bootstrapping was set to 5,000 iterations with a confidence interval of 95% for hypothesis testing. The results analyzed through PROCESS macro Model 1 are presented in Table 7. The analysis revealed that both the independent variable, entrepreneur's age, and the moderating variable, positive gains after failure, significantly impacted the dependent variable, serial entrepreneurship speed (B = -.427), which is consistent with the OLS analysis results.

Table 7. PROCESS Macro Model 1 Results

		B	SE	t	p	LLCI	ULCI
Constant		4.563	.608	7.501	.000	3.356	5.770
Direct Effect	Entrepreneur's Age (A)	.377	.150	2.506	.013	.078	.675
	Positive Gains After Failure (B)	.183	.255	.719	.473	-.323	.690
Moderating Effect	A × B	-.427	.207	-2.065	.041	-.838	-.016
Control Variables	Economic Family Support	-.062	.047	-1.302	.195	-.157	.032
	Psychological Family Support	-.203	.093	-2.173	.032	-.389	-.017
	Self-efficacy	.173	.092	1.878	.063	-.009	.355
	Cause of Failure	-.159	.086	-1.842	.068	-.330	.012
	Duration of Operation	-.327	.095	-3.409	.000	-.517	-.136

To verify the dual moderation effect of positive gains after failure on the relationship between the entrepreneur's age and serial entrepreneurship speed, the results of the Johnson-Neyman technique analysis are presented in Table 8. The analysis revealed that when positive gains after failure are high, the effect is statistically significant (b = .377, CI: [.078, .675]).

Table 8. Analysis of the Moderating Effect of Positive Gains After Failure

Positive Gains After Failure	Effect	SE	t	p	LLCI	ULCI
Low	-.050	.142	-.355	.722	-.332	.231
High	.377	.150	2.506	.013	.078	.675

In Figure 2, it can be observed that when positive gains after failure are high, the serial entrepreneurship speed significantly increases. Conversely, in cases where positive gains after failure are low, there is no noticeable change in the serial entrepreneurship speed.

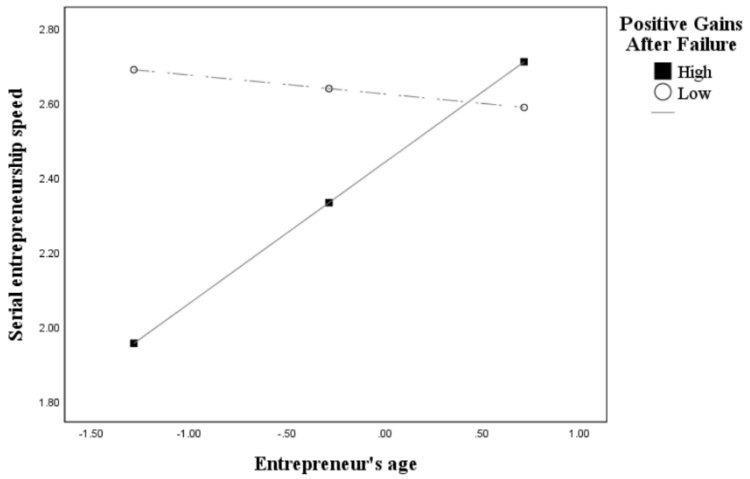

Fig. 2 Analysis of the Dual Moderating Effects of Positive Gains After Failure

5. CONCLUSION

5.1. RESEARCH FINDINGS

This study focused on three key factors in the entrepreneurial process: the entrepreneur's age, serial entrepreneurship speed, and positive gains after failure. It emphasized the definitions and characteristics of serial entrepreneurs, particularly examining how the entrepreneur's age significantly impacts serial entrepreneurship speed. Various causes of failure and family support variables were integrated as control variables for analysis. Thus, this study provided an in-depth discussion on the behavioral characteristics of entrepreneurs who have experienced business failure, as well as their learning and recovery processes.

The primary goal of this research was to verify the impact of the entrepreneur's age on serial entrepreneurship speed and to assess the moderating effect of positive gains after failure in this relationship. Data were collected from entrepreneurs who had experienced business failure, and the moderated moderation model was tested using the Hayes PROCESS macro analysis method. The summary of the findings is as follows:

1. Effect of Entrepreneur's Age on Serial Entrepreneurship Speed:

The analysis of the main effects of variables influencing serial entrepreneurship speed revealed that the entrepreneur's age significantly affects this speed. This indicates that the entrepreneur's age is a critical factor in the serial entrepreneurship process. Older entrepreneurs tend to have more experience, enabling them to make more careful and effective decisions. Therefore, considering the entrepreneur's age when developing and implementing serial entrepreneurship strategies is crucial. These results could enhance the understanding of serial entrepreneurship and aid decision-making in actual serial entrepreneurship processes.

2. Role of Positive Gains After Failure:

The study concluded that positive gains after failure strengthen the relationship between the entrepreneur's age and serial entrepreneurship speed. This suggests that positive gains have a

greater impact on older or more experienced entrepreneurs, facilitating a faster serial entrepreneurship speed. Consequently, both the entrepreneur's age and positive gains after failure can act complementarily in influencing serial entrepreneurship speed.

5.2 IMPLICATIONS

First, it was confirmed that the entrepreneur's age has a significant impact on the speed of serial entrepreneurship. In particular, older entrepreneurs tend to be more experienced, allowing them to make more careful and effective decisions. This suggests that age is an important factor in the serial entrepreneurship process. These results emphasize that entrepreneurs should consider their age when developing and implementing strategies for serial entrepreneurship, which could aid decision-making in actual scenarios.

Second, the analysis indicates that positive gains after failure strengthen the relationship between the entrepreneur's age and serial entrepreneurship speed. Specifically, positive gains after failure appear to have a greater impact on older or more experienced entrepreneurs. This suggests that these entrepreneurs are more likely to view failure positively and learn from it, thereby enhancing their serial entrepreneurship speed. Thus, the entrepreneur's age and positive gains after failure can act complementarily to promote serial entrepreneurship speed.

In summary, there is a need to develop entrepreneurial support policies that take into account the characteristics of entrepreneurs by age. Specifically, policies should be strengthened to support older entrepreneurs by leveraging their experience and self-efficacy.

5.3 LIMITATIONS AND FUTURE RESEARCH

While this study investigated various factors affecting the speed of serial entrepreneurship, it has several limitations:

First, the limited sample size restricts the generalizability of the research findings. A study with a larger number of participants is needed to obtain more accurate and reliable results.

Second, the analysis was conducted based on a limited dataset, which may constrain the applicability of the results. Future research should aim to expand the understanding of serial entrepreneurship speed across diverse cultural, industrial, and geographic contexts.

Finally, although this study aimed to control for other factors affecting serial entrepreneurship speed using control variables, it was challenging to account for all potential variables comprehensively. Therefore, future research should consider and analyze a broader range of factors that may influence serial entrepreneurship speed.

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