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Unveiling the Thermostability, Safety, and In vitro Delivery of Starch-Coated Enriched Palm Kernel Cake Synbiotic Pellets with Immobilized *Lactiplantibacillus plantarum* ATCC 8014

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ABSTRACT

The innovative development of pelleted synbiotic feed additives has increased animal productivity. However, high pelleting temperatures during industrial production would compromise the viability of probiotics, as they are thermally sensitive. This study aims to develop the best pelleted starch-coated enriched Palm Kernel Cake (PKC) with immobilized Lactiplantibacillus plantarum ATCC 8014 for preserving probiotic survivability, analyzing physicochemical, thermal, toxicity, and in-vitro probiotic release. A 2×3 factorial experiment was designed involving 2 pelleting temperatures [60°C (T1) and 70°C (T2)], and 3 additive percentages [1% (A1), 2% (A2), and 3% (A3)]. The results show that T1A2 (2% additive, pelletized at 60°C) had the highest probiotic survivability. Increasing pellet hardness led to reduced moisture content and water activity. FT-IR analysis indicates that T1A2 closely resembles the control pellet, with a slight peak shift at 3500–3300 due to additives and feed interaction. Toxicity analysis confirms safe levels of Aflatoxin (<20 µg/kg) in pelleted L. plantarum. T1A2 had a high decomposition temperature and a 77°C melting point. In vitro release analysis in ruminant rumen maintained probiotic survivability until reaching the target release point. Heterogeneous encapsulant matrices enhance L. plantarum survivability by adding heat insulation within pellets. The best feed additive formula involves pelleting at 60°C with 2% additive, ensuring continuous delivery and maximizing probiotic survival for better health benefits.

1. Introduction

Pelleting in feed processing compresses ingredients through die apertures with steam, heat, and pressure, enhancing nutrient digestibility, reducing waste, and improving feed quality[1]. Feed

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manufacturers employ various combinations of conditioning temperature and retention duration in this process[2]. Some feed mills now reach conditioner temperatures exceeding 90°C during pelleting [3]. Research suggests that adding probiotics like Lactiplantibacillus sp. and yeast to pellet feed faces challenges due to their sensitivity to high temperatures during manufacturing, affecting their viability and stability, despite their potential to boost animal growth and productivity[4,5]. Heat may denature important proteins and enzymes, limiting bacterial viability [6,7]. Research has been conducted on the development of thermophilic probiotic bacterial strains, protective chemicals, and coatings that improve resistance to stresses encountered [8].

Microencapsulation effectively shields probiotics from thermal stress [9]. Kumaree et al. [10] studied L. plantarum encapsulation with varying alginate and fish gelatine protein concentrations to improve its protection and growth in fish feed pellets. Recently, Wang et al. [11] studied broiler feed pellets (BFPs) with probiotics, using direct spraying of L. salivarius NRRL B-30514 solution with varying milk powder and sucrose concentrations, followed by drying at 60°C for different durations. Amerah et al. [12] emphasized the need for additives that withstand harsh feed processing. Shaharuddin and Muhamad [13] found that pre-immobilization of probiotics improved microencapsulation efficacy and cell viability during heat exposure at 90°C, with significantly higher survival rates for immobilized L. rhamnosus (55–56%) compared to free-cell L. rhamnosus (32%) after 30 seconds.

Incorporating coating polymers and prebiotics outperform free-state bacteria preservation across various conditions. Zanjani and colleagues [14] noted that a 60°C heat treatment for 30 minutes reduced unbound cells by about 5 log cycles, while encapsulated L. acidophilus LA-5 decreased by only 1.99 log cycles. Shaharuddin et al. [15] support this, highlighting the significant potential of heterogeneous material synthesis for producing heat-resistant probiotic microcapsules. The concentration of the encapsulation wall can impact the material's thermal conductivity. Statistical analysis demonstrated that microencapsulated cells were significantly more heat-resistant than unstructured cells. L. rhamnosus exhibited a survival rate of up to 4.5 log CFU/g, achieving 81.30% cell viability in microcapsules made with 3% sodium alginate and a 1:1.5 sugarcane bagasse to sodium alginate ratio [13].

Previous research on probiotic survival, physicochemical traits, in-vitro release, and thermal tolerance with pelleted PKC and starch-coated immobilized L. plantarum ATCC 8014 has been limited. The objectives of this study are to determine the survivability of immobilised L. plantarum in starch-coated enriched PKC during pelleting, as well as to analyse the physicochemical, thermal, storage, survivability, toxicity, and in-vitro release of pelleted L. plantarum in a simulated rumen stomach using an animal model. In this study, we immobilized probiotics, primarily L. plantarum, with fiber-rich PKC, serving as both a prebiotic and creating a synbiotic effect to potentially prolong probiotic shelf life.

2. Materials and Methods

2.1 Preparation of Pelleted Starch-coated Immobilised Probiotic Enriched PKC

The starch-coated immobilized Probiotic-PKC beads were prepared following Mat Ropi.16 Ruminant pellets were made using Super Goat Feed from P&G Agro Vet, Malaysia. Around 1% of dried beads was mixed with raw materials and 15% molasses in a 5 L benchtop mixer (Berjaya Steel, Malaysia) at 200 rpm for 2 minutes. The mixture was then pelleted using a 6 mm die ring in a pellet mill machine (Sunwins Power, Malaysia) with 3 kW power, producing 70 to 100 kg/hour. After cooling, pellets were stored in an air-tight container at 27 ± 1 ºC. Pelleting used a 2 × 3 factorial

design, varying pelleting temperatures (60°C and 70°C) and additive percentages (1%, 2%, and 3%), as listed below:

Control T1	Free probiotic bacteria subjected to pelleting process at 60^{o}C
Control T2	Free probiotic bacteria subjected to pelleting process at 70^{o}C
Control S	Commercial pellet
T1 A1	Immobilized probiotic beads + 1% additive subjected pelleting process at 60^{o}C
T1 A2	Immobilized probiotic beads + 2% additive subjected pelleting process at 60^{o}C
T1 A3	Immobilized probiotic beads + 3% additive subjected pelleting process at 60^{o}C
T2 A1	Immobilized probiotic beads + 1% additive subjected pelleting process at 70^{o}C
T2 A2	Immobilized probiotic beads + 2% additive subjected pelleting process at 70^{o}C
T2 A3	Immobilized probiotic beads + 3% additive subjected pelleting process at 70^{o}C
S1	Starch-coated immobilized probiotic beads + additiv
S2	Uncoated immobilized probiotic beads + additive

2.2 Determination of Probiotic Survivability During Pelleting

The probiotic survivability of L. plantarum in pellet was carried out based on Wirunpan et al. [17] using the following Equation (1):

Probiotic Survivability During Pelleting (%)= (Nrp)/(Nip) ×100 Equation (1)

Nrp:Number of microencapsulated cells released from the pellet (Log CFU/g)
Nip: Number of initial microencapsulated cells in the pellet (Log CFU/g)

2.3 Physiochemical Properties Analysis

The pellet hardness was measured in triplicates at RT (27 ± 2 °C) by using a durometer hardness tester (Durotech, Australia). Water activity of pelleted feed (1 g) was determined by using bench-top water activity meter (Freund, Japan). The moisture content of sample was measured by using moisture analyzer (Freund, Japan). FT-IR analysis was started with sample ground and mixed with potassium bromide (Orioner, Malaysia) at a ratio of 1:100. The peak of each sample was scanned by using a FT-IR Spectrometry (Perkin-Elmer, USA) by averaging 16 scans per sample with a resolution of 4 cm^{-1} and in the range of 4000 to 400 cm^{-1}.

2.4 Pellet Ultrastructure Morphology

Morphological properties were analysed using Scanning Electron Microscopy (SEM) (Hitachi, Japan) at 15 kV, capturing surface and cross-section images at 400X magnification. Surface imaging,

2D/3D, and roughness were assessed via Atomic Force Microscopy (AFM) with an NSG01 probe, scanning a 30.0 µm × 30.0 µm area.

2.5 Thermal Properties Analysis

The thermal properties were determined from Rojek and Wesolowski [18], using Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC 2) (Mettler Toledo, Switzerland). The mechanism was explained using data from probiotic survivability, morphology, and thermal properties, represented by a schematic thermal distribution diagram.

2.6 Toxicity Analysis of Total Mycotoxin and Aflatoxin B1, B2, G1, and G2

Total mycotoxin and Aflatoxin B1, B2, G1, and G2 was quantified in two optimal samples (Control T1 and T1A2) using Enzyme-linked immunosorbent assay (ELISA) (r-biopharm, Germany) and High-Performance Liquid Chromatography (HPLC) (Agilent, USA), which was adapted from Lee and Rachmawati [19].

2.7 Analysis of In vitro Release Using Animal Model

The in vitro release analysis was performed based on method described by Li et al. [20]

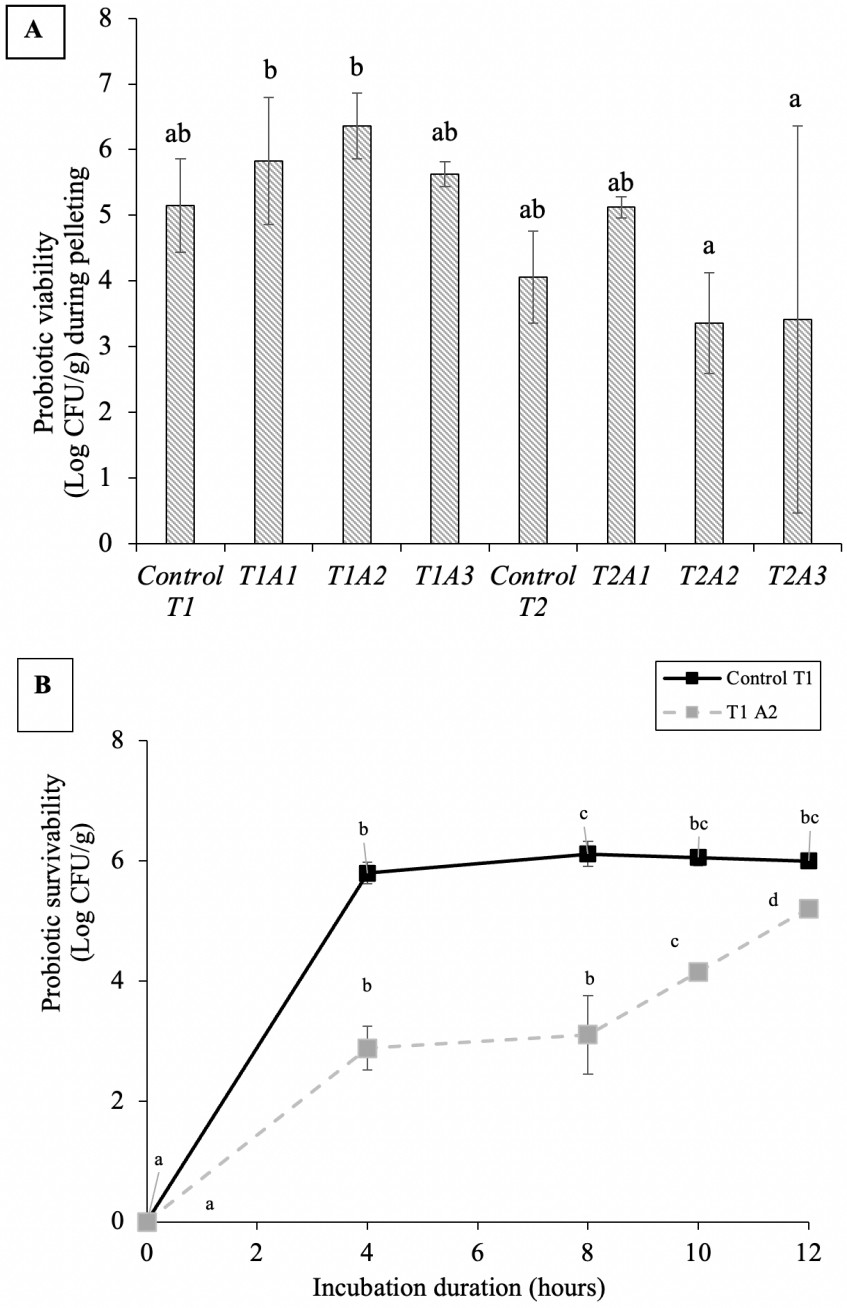
2.8 Statistical Analysis

Data analysis was performed using One-way ANOVA and significance of the results were evaluated using Duncan's multiple range test (DMRT), with was significant at $p < 0.05$. All the samples analysis were performed in triplicates.

3. Results and Discussion

3.1 Survivability of L. plantarum in Pelleted of Starch-coated Enriched-PKC During Pelleting and In Vitro Release

After pelleting at 60°C, the 2% probiotic sample (T_1A_2) showed the highest viability at 6.36 ± 0.50 log CFU/g, while the 2% additive pelletized at 70°C (T_2A_2) had the lowest viability at 3.36 ± 0.77 log CFU/g (Fig. 1A). The maximum quantity of additive seems to be 2% since the viability of the probiotic is compromised when 3% additive is included at a pelleting temperature of 60°C. The potential explanation for this phenomenon might be attributed to the negative impact resulting from the increased number of additives used. This is evident as a larger percentage of additives led to a decrease in the inclusion of additive in the pellet. This was due to the maximum volume capacity of pellet mixture in the selected die ring of the pelleting machine was achieved with 2% additive. Meanwhile, pelleting with 3% additive has contained excessive additive amount that been uncaptured during the pelleting.



ig. 1. Probiotic viability (mean ± S.D.) of immobilised-probiotic-PKC bead at various pelleting temperature and additive percentages (A), and In vitro release of pelleted L. plantarum (B). Different letter (a,b,c) on the line is significantly different ($p < 0.05$).

It is pertinent to emphasize that the immobilization of probiotics and PKC via the encapsulation process using alginate and starch resulted in the formation of a heterogeneous encapsulant. This encapsulant effectively protected the probiotics from direct heat exposure during the pelleting process. Based on the findings of Ma et al.[21], it was observed that the growth rate of *L. plantarum* KLDS 1.0628 exhibited a minor decline at heat treatment temperatures of 40°C and 45°C, with no significant impact on the viable counts in comparison to untreated cells. However, when the temperature surpassed 50°C, the rate of cell growth experienced a substantial reduction of over 95% following a one-hour exposure period [21]. The growth rate of bacteria is mostly hindered by

sub-lethal heat treatment, which induces heat stress leading to notable protein denaturation, cell wall impairment, and damage to nucleic acid molecules [22]. Furthermore, this additive demonstrates the capacity to safeguard probiotic cells from the detrimental effects of heat treatment. The survival of the unprotected probiotic cell was found to be only 10.48 ± 18.16%, in contrast to the current study's findings. The importance of suitable selection of material for immobilization is critical as it can act as energy sources in the form of carbon and nitrogen to improve activity or growth of probiotic [23].

In vitro release analysis shows that *control* T_1 exhibited the highest probiotic release, measuring 6.12 ± 0.21 log CFU/g after 8 hours. In contrast, T_1A_2 achieved a probiotic release of 5.21 ± 0.04 log CFU/g after 12 hours (Fig. 1B). Conversely, the initial 4-hour period of release exhibited the lowest levels of probiotic release for the *control* T_1 and T_1A_2 samples, measuring 5.80 ± 0.18 and 2.89 ± 0.36 log CFU/g, respectively. The alginate material exhibited swelling behavior upon contact with water, resulting in the expansion of the microcapsule's pores and subsequently leading to a decelerated release rate. The probiotic included in sample T_1A_2 necessitates traversing layers of alginate and starch encapsulants prior to its release. Consequently, the substance exhibits a gradual release mechanism until it reaches the intestinal region. Encapsulation using protective materials provides several benefits, including protection, controlled release, and improved distribution. Therefore, this technique enables the targeted administration of probiotics to the desired site of action [24]. The second stratum, referred to as the starch coating in the context of this study, enhances the beads' resilience under acidic conditions. The beads containing the second layer are discharged within certain pH conditions at designated sites [25]. This approach enhances the *in vitro* survival of probiotics. Significant changes $(p < 0.05)$ occurred at 0 and 4 hours for sample *control* T_1, but not at 8, 10, and 12 hours. Sample T_1A_2 displayed significant differences $(p < 0.05)$ at 0, 10, and 12 hours, with no differences $(p > 0.05)$ at 4 and 8 hours in this study. After reaching its peak release at 8 hours in *control* T_1, the subsequent probiotic release was minimal. Similarly, in the T_1A_2 experiment, analysis at 4 and 8 hours showed probiotic regulation, but the effect was statistically insignificant.

In this study, sample T_1A_2 showed improved probiotic viability after encapsulation and pelleting. According to Iommelli et al. [26], feed's nutritional composition depends on nutrient content, rumen degradation, and digestibility in the small intestine. Therefore, it is crucial to guarantee the successful delivery of the safeguarded probiotic to the specific location of the small intestine in order for the host animal to obtain its advantageous effects. Furthermore, it is anticipated that the constituents and compositions of the pelleted feed will have an impact on dissolving, as pelleting alters the metabolic and digestive properties of the feeds [27]. Pelleted feed may take longer to fully disintegrate the added feed additive. Adjei-Fremah et al. [28] emphasized that probiotics target the rumen in ruminants, affecting fermentation, feed digestibility, degradability, and the rumen microbiota composition. Uyeno et al. [29] proposed that probiotics, such as LAB species, frequently exhibit a preference for the lower intestine in young pre-ruminants. This preference serves the purpose of preserving the gut microbiota and mitigating the risk of pathogen colonization. Nevertheless, the results obtained for the T_1A_2 sample (60°C pelleting, 2% additive) indicate a progressive increase in the release rate, with two distinct release events occurring at 4 and 8 hours. This finding illustrates that the probiotic remained shielded and retained its viability throughout the entirety of the research.

3.2 Physiochemical Properties and Toxicity Analysis of Pelleted L. plantarum

The hardness analysis revealed that T_2A_1 (1% additive, 70°C pelleting) had the highest hardness at 91.72 ± 0.35 (Table 1), followed by the *control* T_1 at 91.22 ± 1.61 (60°C pelleting). T_2A_2 (2% additive,

70°C pelleting) had slightly lower hardness at 91.06 ± 0.42, while pelleting at 60°C with a 3% additive resulted in the lowest hardness at 83.06 ± 2.71. As the additive amount increases, pellet hardness decreases, affecting the pellet durability index [30]. Low-hardness pellets tend to disintegrate quickly, causing particulate matter and disruptions in feeding systems [31]. Pelleting at 70°C reduced the impact of higher additive proportions by enhancing water evaporation, increasing pellet hardness.[32] Higher temperatures also promoted gelatinization, strengthening the link between temperature and hardness [32]. Overall, higher pelleting temperatures can affect evaporation rates, potentially influencing sample hardness. Pelleting at 70°C results in slightly harder pellets compared to 60°C pelleting.

Table 1
Physical properties of pelleted L. plantarum and correlation among matrix of parameters

Sample	Hardness (mean ± S.D.)	Water Activity (A (mean ± S.D.)	Moisture Content (mean ± S.D.)
Control T_1	91.22 ± 1.61^{c}	0.6289 ± 0.09^{a}	6.12 ± 1.58^{a}
T_1A_1	88.33 ± 1.73^{bc}	0.6443 ± 0.10^{a}	7.45 ± 2.03^{a}
T_1A_2	86.83 ± 3.88^{b}	0.6508 ± 0.13^{a}	7.80 ± 3.29^{a}
T_1A_3	83.06 ± 2.71^{a}	0.6521 ± 0.06^{a}	8.55 ± 4.27^{a}
Control T_2	89.54 ± 0.70^{bc}	0.6380 ± 0.02^{a}	7.21 ± 1.46^{a}
T_2A_1	91.72 ± 0.35^{c}	0.5927 ± 0.01^{a}	4.39 ± 0.97^{a}
T_2A_2	91.06 ± 0.42^{c}	0.6080 ± 0.03^{a}	5.06 ± 0.44^{a}
T_2A_3	89.83 ± 1.30^{bc}	0.6093 ± 0.01^{a}	5.24 ± 0.61^{a}

^{abc}Values with different superscript letter within a column were significantly different *($p < 0.05$).*
^{a}Values with different superscript letter within a column were not significantly different *($p > 0.05$).*

	Hardness	Water Activity	Moisture Content
Hardness	1	-0.366	-0.536**
Water Activity		1	0.534**
Moisture Content			1

**Pearson correlation was significantly different at $p < 0.01$.
Number of samples, N = 24.

The water activity values for all treatments ranged from 0.59 ± 0.01 to 0.65 ± 0.06, and the moisture content varied between 4.39 ± 0.97 and 8.55 ± 4.27. Increasing the additive quantity inside the pellet at 60 °C led to a slight rise in water activity. Lambert et al. [33] found that the water activity of mixes is influenced by component fraction size, particularly the additive proportion in this study. Cheah et al. [34] emphasize the importance of low water activity levels, as pathogenic

bacteria thrive between 0.995 and 0.980, while some yeasts and molds may flourish below 0.60. Raising the pelleting temperature reduces pellet water activity. This may stem from feed water activity changes influenced by moisture content and storage temperature [35]. At 70 °C, higher evaporation rates further lower water activity.

To maintain pellet longevity without water absorption, high water impermeability is crucial [36]. At 60°C, higher additive percentages led to increased moisture content, likely due to the additive's higher alginate, PKC, and starch content, which retain more moisture. However, at 70°C, the pellet temperature reduced moisture induced by the additive when exposed to higher heat. Ungureanu et al. [36] suggested a 4-month storage moisture range of 11% to 13% for pellets. Wirunpan et al. [17] found that drying at 50, 60, 70, and 80 °C lowered moisture below 11%, meeting probiotic product requirements, possibly due to thermal energy effects. Higher temperatures boost water molecule energy, enhancing digestibility and destabilizing proteins, as suggested by Oliveira et al. [37] However, elevated moisture impedes water removal during pellet compression, causing volume expansion and density reduction [32]. The correlations presented in Table 1 provide support for the current findings, which suggest a moderate inverse relationship between moisture content and pellet hardness (R = -0.536; $p < 0.01$). Additionally, a positive relationship is observed between moisture content and water activity (R = 0.534; $p < 0.01$).

3.3 Chemical Composition by FT-IR

Fig. 2A illustrates the primary raw ingredients used in the process, namely PKC, *L. plantarum*, alginate, starch, and S1. The peak seen at 3442 cm^{-1} in the palm kernel cake spectrum is attributed to the stretching vibrations of hydroxyl (O-H) functional groups in polysaccharides. The existence of the -COOH functional group is indicated by the peak seen at 2363 cm^{-1}, as reported by Hamza et al. [38] The presence of a double bond between carbon and oxygen (C=O) in fatty acids with high lipid content, such as palmitic or oleic acid, has been identified by the observation of a peak at 1645 cm^{-1}. [39] The observed peak at 1067 cm^{-1} may be attributed to the stretching vibration of C-C, C-O, and C-O-C bonds, which are often associated with the presence of sugar aldehyde groups [40]. The FT-IR spectra of *L. plantarum* featured a prominent peak at 3303 cm^{-1}, signifying extensive O-H group vibration related to the carbohydrate ring. The 2932 cm^{-1} signal indicated aliphatic CH_2 groups found in proteins and organic substances. A 1655 cm^{-1} peak, as identified by Elova et al. [41], indicated prolonged C=O functional group vibration. The 1046 cm^{-1} peak, identified by Mohd Yusof et al. [42], was linked to hydroxyl groups in saccharides (C-O bond). Alginate encapsulation, following immobilization of PKC and *L. plantarum*, exhibited OH group (3421 cm^{-1}) and -CH vibration (2927 cm^{-1}) peaks, as described by Badita et al. [43] Starch coating minimally shifted the peak at 1463 cm^{-1} for the C=C aromatic group. Beads (S_1) showed peaks similar to other raw materials.

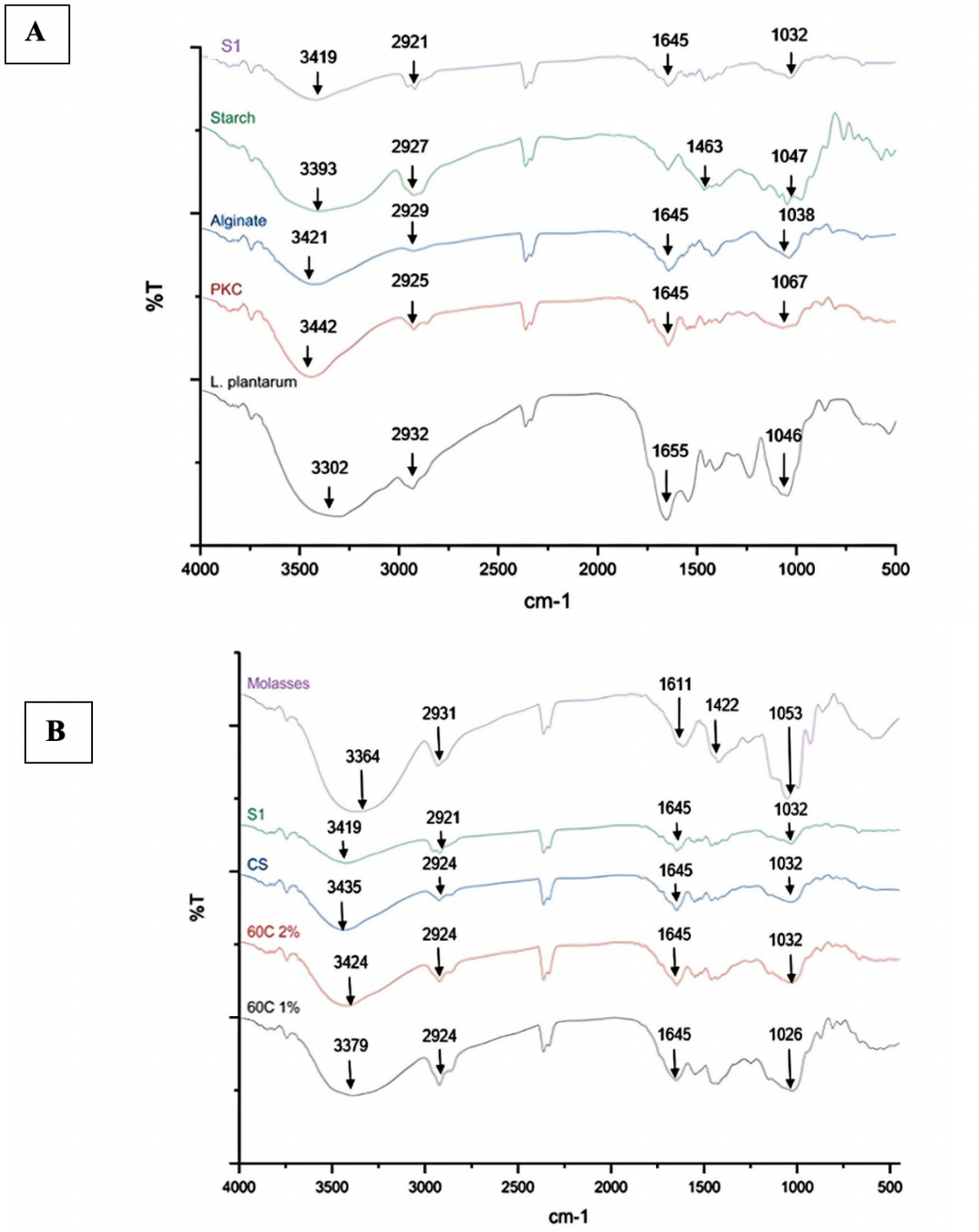


Fig. 2. FT-IR spectra of starch-coated-probiotic immobilized PKC containing *L. plantarum*, PKC, alginate, starch, and S_1 (A) and pelleted *L. plantarum* consisting of T_1A_1, T_1A_2, *Control S*, S_1, and molasses (B)

Fig. 2B depicts the FT-IR spectra of the various elements included in the pelleted *L. plantarum*. These materials include the additive bead (S_1), commercial pellet (*Control S*), molasses, T_1A_1 (1% additive, pelleting at 60°C), and T_1A_2 (2% additive, pelleting at 60°C). The observed wavenumbers for the O-H band in *Control S*, T_1A_1, T_1A_2, and molasses were found to exhibit shifts of +16, +5, -40, and -55 cm^{-1}, respectively. The observed shift in the spectrum peaks for T_1A_1 and T_1A_2 may be attributed to variations in the amounts of the additive used. Additionally, this phenomenon may be attributed to the interplay between the feed mixture and molasses. The CS, T_1A_1, and T_1A_2 peaks seen at the CH, C=O, and C-O bands exhibited little or negligible changes. In the case of molasses, a significant

displacement of the C=O band was seen at a wavenumber of 1611 cm^{-1}. A novel spectral peak emerged with a wavenumber of 1422 cm^{-1}, indicative of the presence of supplementary alkene and aromatic moieties (C=C). The observed shift in the peak of C-O stretching at 1052 cm^{-1} may be attributed to the increased concentration of polysaccharides. Overall, based on S_1 findings, the pelleting process minimally altered the peak distribution for *CS*, T_1A_1, and T_1A_2.

3.4 Ultrastructure Morphology of Immobilized-probiotic-PKC beads

Table 2 displays pellet images at 400X magnification, showing differences in structure due to feed combinations, pelleting temperature, and additive content. Commercial pellet *(Control S)* appears rougher and less structured. Higher additive concentrations yield smoother surfaces and fewer holes at 60 °C, while 70°C with increased additives leads to denser pellet surfaces due to additives within pores. The cross-sectional images reveal that pellets with 2% additive exhibit tighter packing than those with 1% additive, as well as control groups T_1 and T_2 (free-cell) and *control S* (commercial pellet). *Control S* displays a narrow line structure, while 1% and 2% additives show broader lines, possibly composed of diverse encapsulant material. The outer surface compactness varies at 60°C and 70°C. The samples pelleted at 60°C had a smoother surface, while those at 70°C were rougher and more crumpled. The pellet with free-cell probiotics appeared rough with well-defined lines, while the one with an encapsulated additive looked uniform, especially at 60°C. However, the structures showed unevenness due to the encapsulant, and the pores varied in size and smoothness on the pellet's surface and inside [44]. This phenomenon may result from a starch coating on the external surface of the additives. Krishnan et al. [45] explored the surface morphology of kenaf pellets, adding starch to improve fiber bonding and pellet durability. Amylose's ability to enclose molecules and form stable complexes might explain its protective effect, as proposed by Muhammad et al. [46].

Results show that *control S* had the lowest surface roughness at 32.62 nm, while T_2A_2 had the highest at 57.02 nm. Pellet roughness correlated positively with both pelleting temperature and additive proportion. Fig. 3 highlights clear differences between *control S* and T_2A_2 in 2D- and 3D-images. This may be due to T_2A_2's production at a higher pelleting temperature (70°C) and a larger additive proportion. These factors likely caused significant surface abnormalities. Unlike T_2A_2, 2D-image of *control S* shows a smoother, more uniform surface. The 3D-image of the sample at 70°C displays more peaks, increasing surface roughness, while *control S* has fewer visible peaks. The pelleting process involves high temperatures to compress the feed into pellets. Higher additive concentration increases pellet compaction, leading to rougher surfaces at 70°C due to elevated temperatures intensifying roughness.

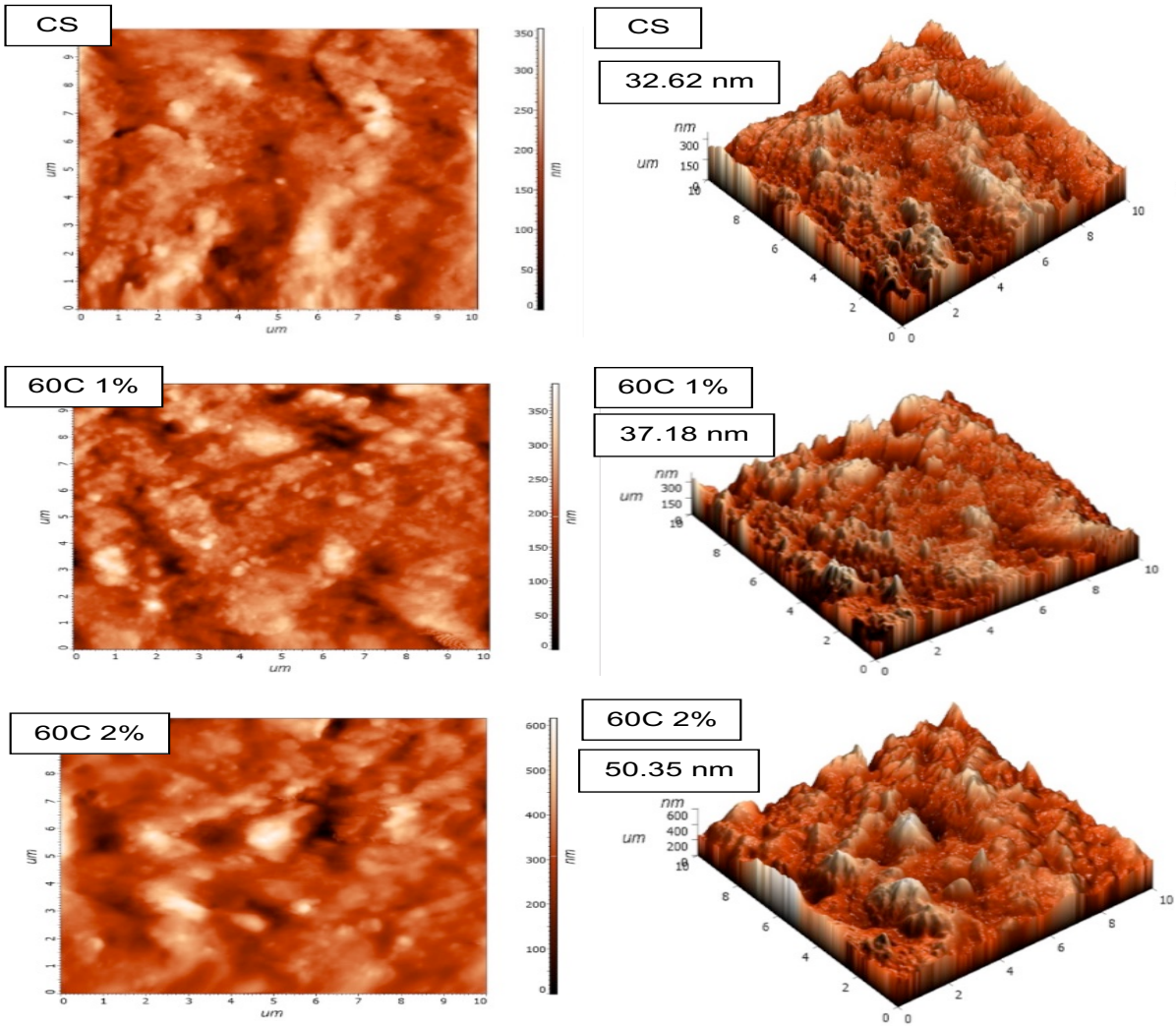


Fig. 3. 2D- and 3D-images of pelleted L. plantarum using AFM

3.5 Toxicity Level

The obtained results indicated Aflatoxin levels of 3.4 and 3.3 µg/kg for control T_1 and T_1A_2, respectively. These values fall below the established limits. During the measurement of Aflatoxin using the ELISA, it was observed that both control T_1 and T_1A_2 samples exhibited Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, and Aflatoxin G2 levels < 1.00 µg/kg. According to the US Food and Drug Administration, the action levels for Aflatoxin M1 are 0.50 µg/kg in liquid milk, 20 µg/kg for total aflatoxins in ingredients used to make feed for dairy cattle, 100 µg/kg for breeding cattle, 300 µg/kg for beef cattle that are finishing, and 20 µg/kg for human food [47]. The metabolism of Aflatoxin B1 occurs in ruminants upon ingestion of food containing this toxin, resulting in the secretion of Aflatoxin M1 in milk [48]. However, the bacteria present in the rumen and the feed particles inside the rumen compartment exhibit a high degree of effectiveness in the process of breaking down, neutralising, and attaching themselves to harmful substances. According to Gallo et al. [49], the use of this measure may enhance the protection of ruminant animals by decreasing their vulnerability to mycotoxins in comparison to monogastric animals.

3.6 Thermal Properties

Fig. 4A presents TGA curves, revealing the temperatures at which the *L. plantarum* sample disintegrated. Specifically, the *L. plantarum* sample disintegrated at 83.45 °C, while the encapsulated bead (S_1) disintegrated at a higher temperature of 116.74 °C. In contrast, the pellet-containing bead (*control S*) disintegrated at a lower temperature of 72.71 °C. Sample S_1, with its heterogeneous encapsulant, showed a delayed initial inflection point at 116.74 °C, while *control S*, with its encapsulant, broke down rapidly at 72.71 °C. This behavior can be attributed to thermal resistance due to alginate and starch, as well as the immobilization of probiotics and PKC. The inflection points on the weight loss curve, representing the moments of maximum rate of change. The probiotic experienced a significant reduction at its initial inflection point at 83.45 °C, leaving a residual amount of 40.42%.

When probiotic samples with additives and starch coating were analyzed, T_1A_2 had the highest decomposition temperature at 285.81 °C, followed by T_1A_1 at 283.97°C, T_2A_1 at 282.95°C, and T_2A_2 at 274.96°C (Fig. 4B). T_2A_2 exhibited an initial inflection point at 274.96°C with a residue of 49.37%, followed by a gradual reduction. *Lactiplantibacillus* sp., Gram-positive bacteria, can withstand temperatures up to 75°C, but probiotics degrade beyond their decomposition temperature due to a specific element in their structure [50]. Unlike pelleted probiotic T_2A_2, the studied sample contains multiple components: probiotic, alginate, PKC, starch, water, molasses, and a feed combination, each with its unique breakdown temperature. Increasing the temperature is necessary for material decomposition. Romuli et al. [51] researched biomass pellet breakdown, observing characteristic decomposition kinetics between 180°C and 500°C. However, there is limited research on the decomposition temperature of probiotic pellets used as animal feed.

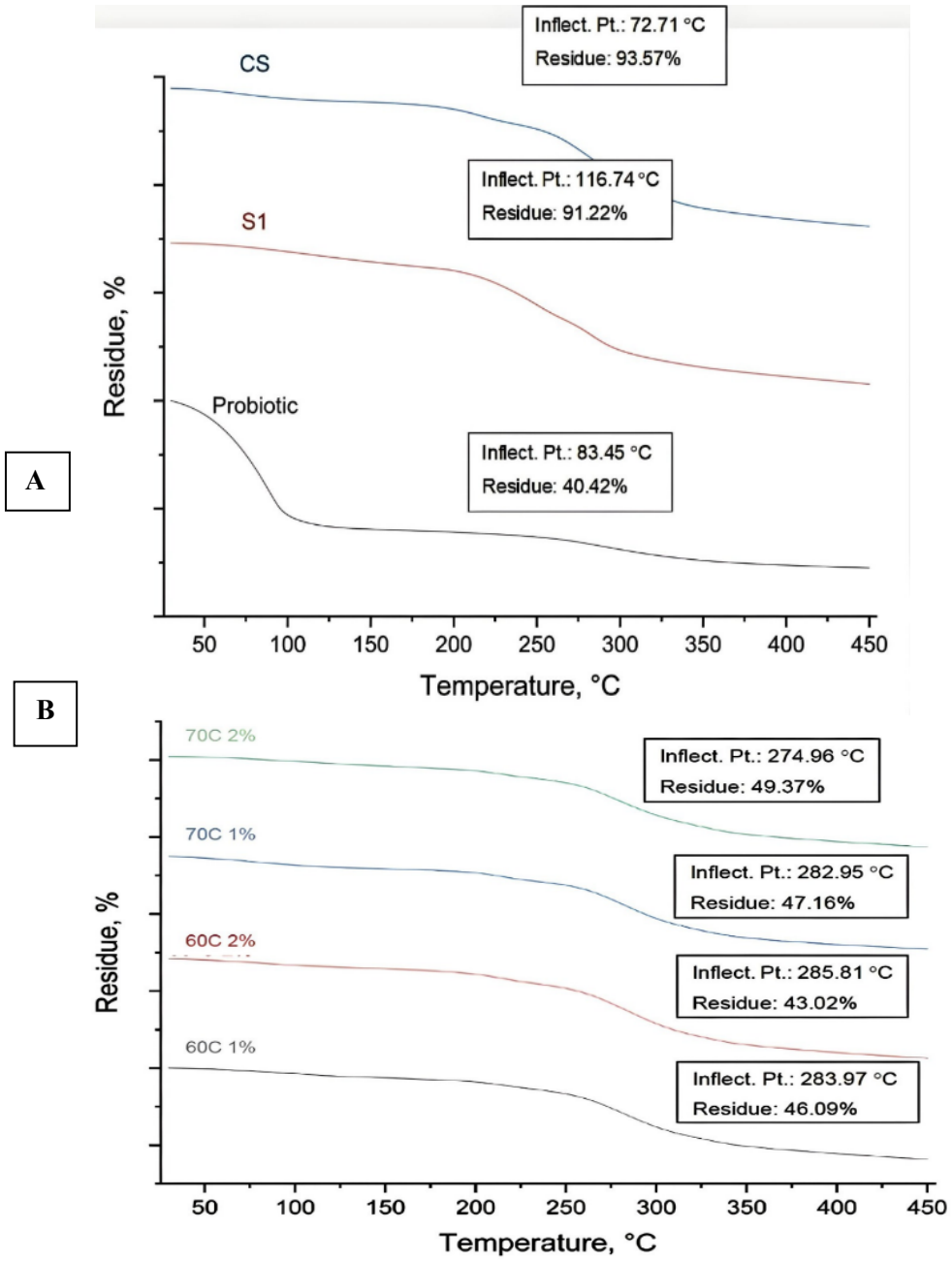
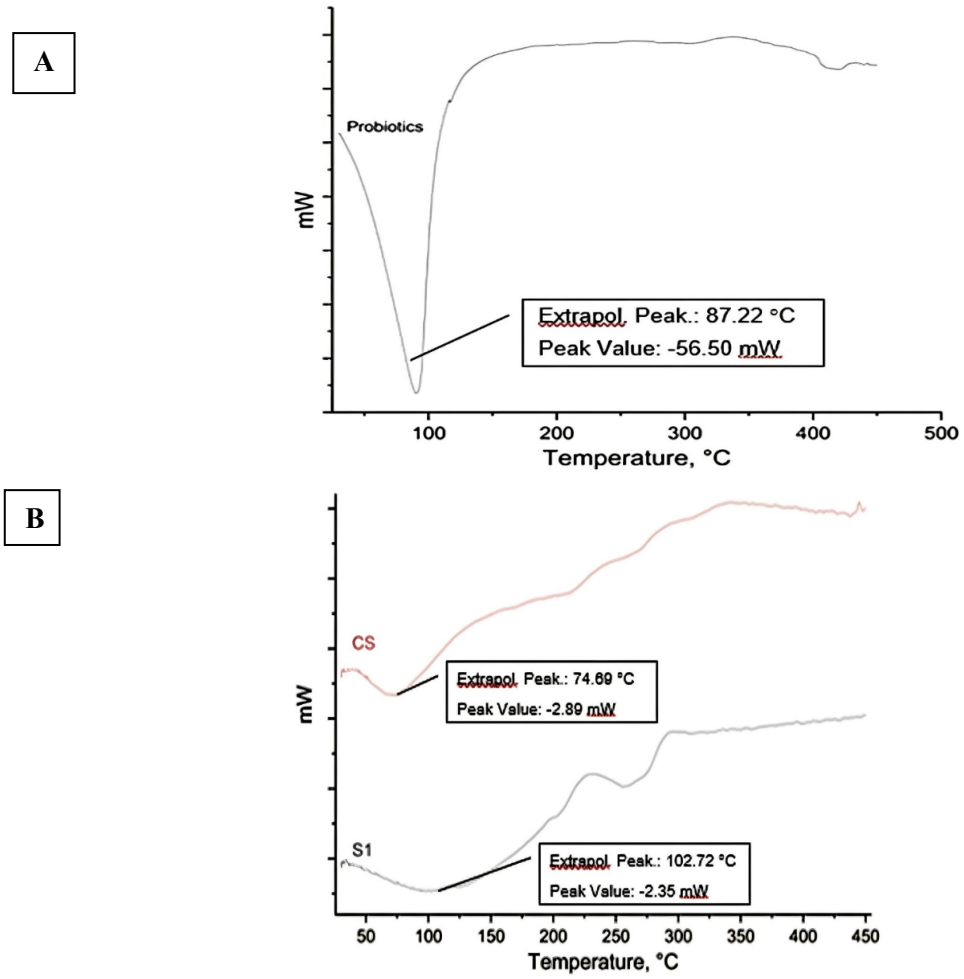


Fig. 4. Thermogravimetric analysis of decomposition temperature of probiotic, additive with starch (*S1),* and commercial pellet (*CS)* (A), and T_1A_1, T_1A_2, T_2A_1, and T_2A_2 (B)

The DSC analysis of a probiotic sample of *L. plantarum* is depicted in Fig. 5A, showing an endothermic peak at 87.22 °C with a peak value of -56.560 mW, indicative of a "melting" process caused by heat absorption. This endothermic reaction occurs when the sample's temperature is lower than the reference materials. All peaks in the DSC study exhibit endothermic responses, dependent on each material's thermal characteristics. As temperature increases, peak values rise until the sample component reaches its melting point. In Fig. 5B, the initial peak occurs at 102.72 °C with a voltage of -2.35 mV, signifying the encapsulated bead's melting. Conversely, the commercial pellet (*Control S*) has a lower melting temperature of 74.69 °C with a voltage of -2.89 mW.

The sample T_2A_2 has the highest melting point at 214.30 °C and a power of 3.90 mW, as shown in Fig. 5C. T_2A_1 samples have a temperature of 79.67 °C and -3.30 mW power absorption. T_1A_2 samples display 76.99 °C temperature and -2.93 mW power absorption. T_1A_1 samples show 84.58 °C temperature and -1.33 mW power absorption. This pattern is consistent in the TGA data, attributed to higher pelleting temperature and more additives. Elevated melting points increase the heat energy required for endothermic processes. Sample T_2A_2 also exhibits a glass transition, seen in abrupt changes in thermodynamic parameters like heat capacity and thermal expansion. This is known as "amorphous transformation," occurring when a substance shifts between solid and liquid states upon heating or cooling [52].



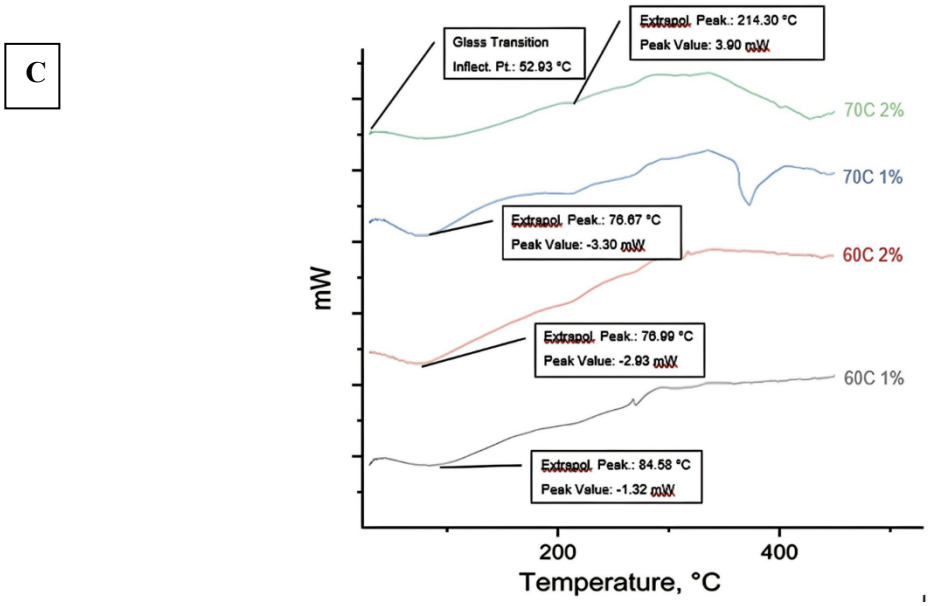


Fig. 5. Melting point of probiotic (A), additive with starch *(S_1)*, and commercial pellet *(CS)*(B) and experimental samples (T_1A_1, T_1A_2, T_2A_1, and T_2A_2) (C)

4. Conclusions

The pelleted probiotic with 2% additive at 60°C (T1A2) showed higher viability (6.36 ± 0.50 log CFU/g) than the 1% additive at 70°C (5.12 ± 0.16 log CFU/g). Increasing additive concentration reduced pellet hardness, increasing water activity and moisture content. T1A2 pellets had higher melting and decomposition temperatures. The pelleted probiotic feed is safe for animals. T1A2 significantly ($p < 0.05$) improved delivery to the small intestine in adverse conditions. L. plantarum pellets protect probiotics from heat exposure. Pelletized T1A2 is the optimal formulation due to its superior properties and heat protection.

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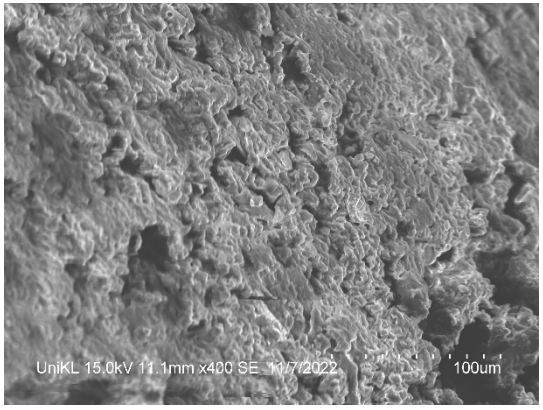
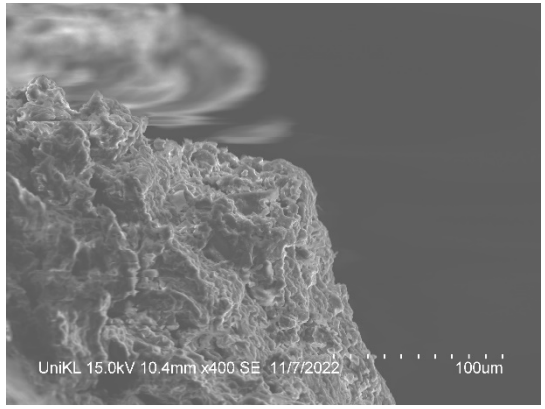
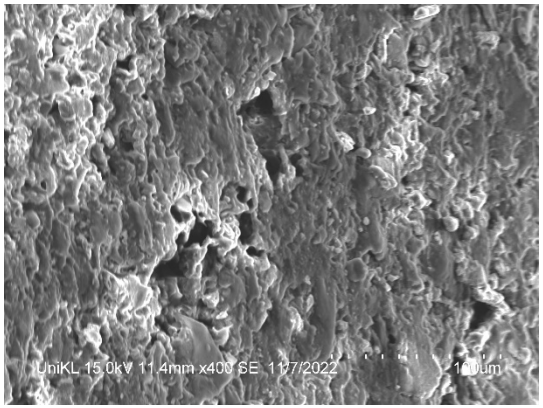
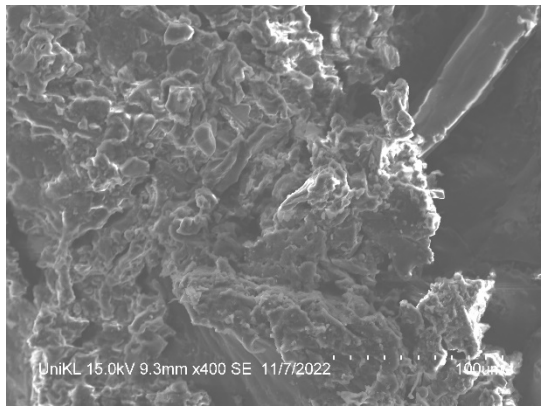
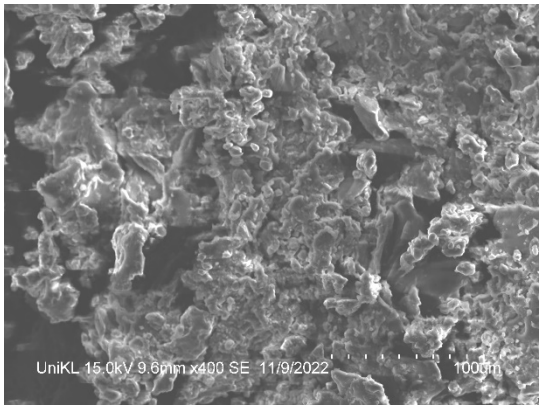
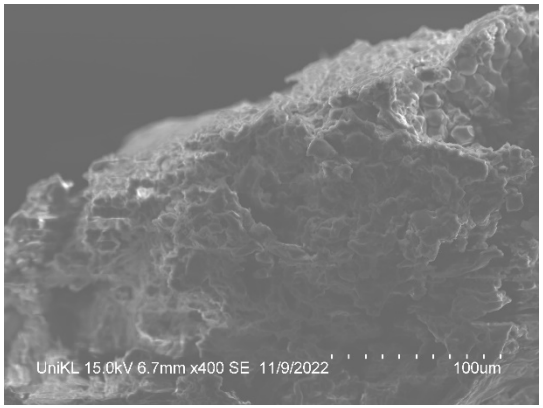
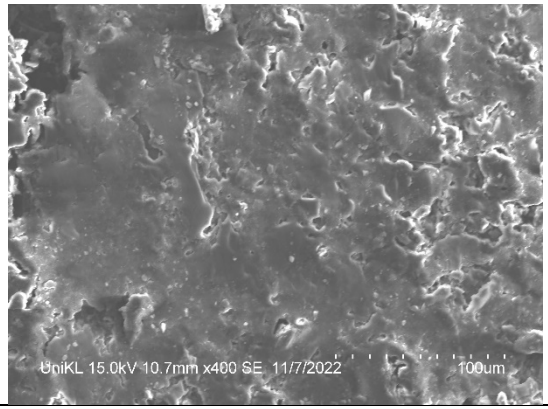
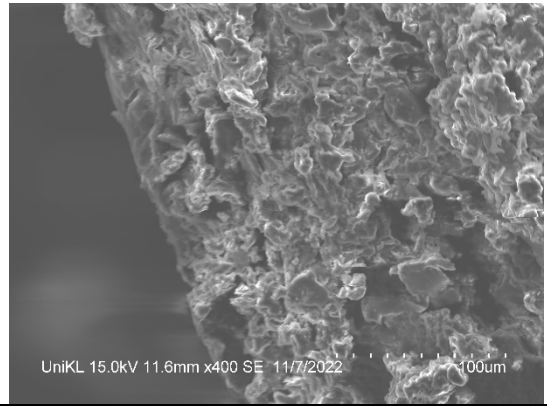
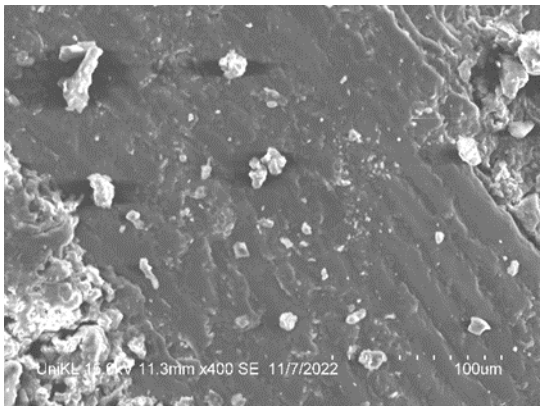
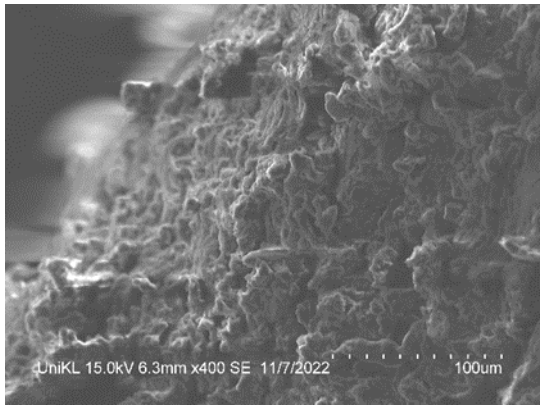
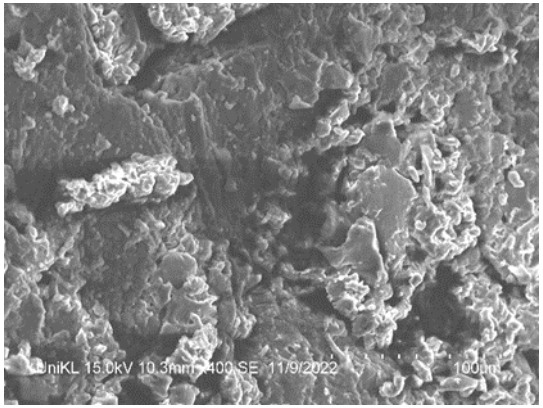
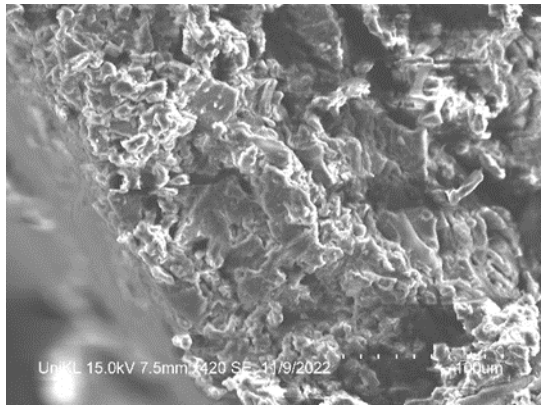
Table 2
Roughness, outer surface and cross-section of pelleted L. plantarum. All the images were captured at 400X magnification

Sample	Roughness average (nm)	Outer Surface	Cross section
Control S	32.62		

Control T_1	-		

Control T_2	-		

T_1A_1	37.18		
T_1A_2	50.35		

T_2A_1	49.56		

T_2A_2	57.02