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# Developing of Edible Protein-Based Films enriched with Saccharomyces boulardii and Bifidobacterium

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**ABSTRACT**

The recent attention has been drawn to edible films and coating since they are low in cost production, and at the same time, they are eco-friendly. These edible materials hold a lot of potential into enhancing food functionality when fortified with probiotics. It was also found that co-administration of probiotics in edible matrices enhances gastrointestinal colonization, probiotic viability and health benefits. The experimental findings showed that the nature and the concentration of the protein to be used in the formulation of the film greatly influenced the mechanical properties. Movies based on 7.5% of both whey protein and casein had a desirable balance between tensile strength and flexibility to that of 5 and 10 percent. Additionally, embedding of Saccharomyces boulardii and Bifidobacterium strains enhanced the performances of the film with the 7.5% protein films with yeast reinforcement showing best results of Youngs modulus (2480 Mpa) and elongation (146%). Likewise, the tensile strength and elasticity of membrane supplemented with Bifidobacterium was also excellent. These results establish that probiotics addition increases the mechanical integrity and structural network of protein-based membranes. On top of such structural benefits, these membranes have nutritional and health advantages that highlight their potential in being used in smart food packaging preserve and enhance food integrity.

# 1-Introduction

Edible protective polymeric coatings and films that are sometimes called edible films and coatings are edible coatings and films applied to the food surface to extend shelf life and they may be eaten along with the product [1]. The presence of protein-based matrices, especially whey protein and casein have gained a lot of popularity because of their excellent film-forming capacity, biodegradability, and compatibility with food usage. Such proteins are cohesive, flexible and transparent films that are formed by intermolecular forces, including hydrogen bonding and disulfide cross-linking, which confer mechanical strength and barrier properties without affecting nutritional or sensorial qualities [2,3]. Besides, protein-based films might serve to provide bioactive compounds and probiotics with a desirable microenvironment, which makes them more stable to the processing and storage. They act as moisture barriers, regulate gas exchange as well as minimise the growth of microbes.

The traditional synthetic plastics are highly harmful to the environment as they are not biodegradable [4,5]. Conversely, edible coatings, particularly those based on microorganisms, act as bio systems that maintain the food quality and are selective in terms of oxygen, carbon dioxide and ethylene exchange between the food and the surroundings [6].

The increased consumer consciousness about the relationship between nutrition and health has seen the creation of the functional foods that are fortified with antioxidants, antibacterial agents, macro and micronutrients, and probiotics [7,8]. Probiotics, by definition, (FAO/WHO) referring to living microorganisms, which, in the correct quantities (10 6 -10 7 CFU/g) provide physiological beneficial properties to the host, are rich in dairy products, cereals, meats, fruits and vegetables because of their fiber content and functional qualities [10,11]. Prebiotics or carbohydrates that cannot be digested can also enhance the survival and stability of probiotics in food and the GIT [12].

There are various advantages of including probiotics in protein-based edible films. The integration of living cells under a thin layer of natural polymer prevents exposure to harsh conditions (thermal, acidic, osmotic and mechanical stress) and preservation (oxygen presence, water vapour permeation) and high intensity delivery to the gastrointestinal tract [13,14]. Bioencapsulation is a more developed process of maintaining live microorganisms at suggested doses, and for improving the viability of probiotics. Furthermore, biopolymers may protect food against environmental conditions, suppress food degradation, and release bio-active substances like vitamins and enzymes [15,16]. Edible coats loaded with probiotics, to be consumed, confer both protective and antibacterial properties, and prebiotics enhance the viability of probiotics, increase the shelf life, and enhance the quality of food [17-22].

In spite of the fact that protein-based edible films and probiotics are investigated separately, there is scarce literature that examines the interaction of various types of proteins and probiotic strains on mechanical properties and functional characteristics of edible films. Vital methodological factors, such as the choice of protein, the probiotic strain, mode of incorporation, and the state of processing, are not frequently reported, which restricts the possibility of reproducibility and practical use. The proposed study will fill these gaps by creating protein-based edible films with Saccharomyces boulardii and Bifidobacterium added, to maximize protein type and concentration, and to assess the mechanical characteristics, probiotic stability, and functional activity of the films as sustainable, health-promoting, food coatings. The findings offer an understanding of the creation of effective functional edible films having higher structural integrity and probiotics delivery.

# 2- Materials and Methods

### 2.1 Preparation of Film Method

The concentration of the protein was used to prepare the film-forming solution to form different levels of 5-10% (w/v) film concentrations, as earlier studies have shown that this level gives rise to edible films which possess desirable mechanical and barrier properties [23]. The choice of protein sources was whey protein isolate and casein because it has a great film-forming capacity and can be used in food preparation.

All of the protein mixtures were stirred and kept on a hot plate until it was thoroughly dissolved and homogenized (2 hours). The solution was subsequently allowed to heat its contents at a temperature of 90degC at a constant rate of

stirring of 30 minutes to cause structural changes that would allow the solution to form films. The pH was then brought to 7.0 with NaOH or 0.1N HCl as necessary after all the sample was cooled to room temperature. Glycerol was presented as a plasticizer in 3 mL of 100 mL of solution.

In the cooled solution, 2 g of freeze-dried probiotic powder was added to enrich it with probiotics. Bifidobacterium bifidum (BIOCODEX, France) and Saccharomyces boulardii (Maxima for Supplements, USA) were used as probiotic strains at a final concentration of an approximate of 1 x 108 CFU/mL. All the components were processed in an aseptic environment and probiotics were added in room temperature without any further culture medium to preserve the viability.

The homogenized solution was poured into 15 cm Petri plates and allowed to dry at room temperature until cohesive films formed. After drying, the films were gently removed and stored in airtight polyethylene bags until further analysis [24]. The most favorable protein concentration for film characteristics was selected for subsequent tests and applications.

**2.2 Mechanical tests for membranes**

**2.2.1 Measurement of the coating thickness:**

Film thickness was measured using a digital micrometer (IDM-D0007, Australia) with 0.001 mm sensitivity. Seven points from the periphery to the center were measured, and the average value was recorded [25].

**2.2.2 Measurement of tensile strength and elongation at break:**

Tensile strength and elongation at break were determined using a Tinius-Oken tensile testing device following ASTM D-882.91 [26]. Films were conditioned at 55% relative humidity for 48 hours, cut into 80 × 20 mm strips, and secured with adhesive tape at the ends. Samples were pulled at 5 mm/s, and stress–strain curves were used to calculate tensile strength and elongation.

**2.2.3 Measurement of the permeability of films to water vapor (Water Vapor Permeability (WVP)**

Water vapor permeability was measured after conditioning films at 55% RH and 23°C for 48 hours [27]. A circular plastic cup (outer diameter 3.1 cm, inner diameter 3 cm, depth 4 cm) containing 10 mL distilled water was sealed with the film. The cup was placed in a desiccator containing sodium bromide at 0% RH and 25 ± 1°C. Weight loss was monitored over 48 hours. WVP was calculated using:

$WVTR=\Delta W\backslash A\times t$

where ΔW is the weight loss of the cup (g), A is the exposed film area (m²), and t is the time (s).

The WVP was then calculated using the equation$=WVTR\times L\backslash\Delta P$

Where ΔW is weight loss (g), A is film area (m²), t is time (s), L is average film thickness (m), and ΔP is the partial water vapor pressure difference (Pa).

**2.2.4 Determination of the solubility of membranes in water (Water Solubility Determination)**

Film solubility in water was determined following [28] with minor adjustments. Samples (2 × 2 cm) were dried at 105°C for 24 hours to determine initial weight, immersed in 50 mL distilled water containing 0.02% sodium azide for 24 hours at room temperature under gentle stirring, then dried again at 105°C. Solubility (%) was calculated based on weight loss.

**2.3 Structural or Spectroscopic Analysis**

**2.3.1 Scanning Electron Microscope (SEM)**

The surface morphology, size and uniformity of particles in films were studied using SEM [29]. This analysis gave data on surface texture, distribution of particles and interactions between components of the film.

**2.3.2 Fourier Transform Infrared spectroscopy FTIR**

The FTIR was applied to determine the functional groups and the interaction among the atoms within the protein films. The spectra were taken at 400-4000 $cm^{-1}$ [30].

**2.3.3 Energy-Dispersive X-ray Spectroscopy (EDX)**

The elemental composition of the films was determined by EDX with the addition of SEM. The sample atoms are excited by the electron beam and produce typical X-rays which are detected to determine the elements in the sample [31].

## 3- Results and Discussion

**Table (4-1) of physical and mechanical properties of whey protein and casein**

| | Protein | Thickness (mm) | Tensila Stress (Mpa) | Young s modulus (MPa) | Elongation at break% |
|---|---|---|---|---|---|
| 1 | WPL 5% | 0.395 | 0.546 | 3.64 | 15 |
| 2 | WPL7.5% | 0.390 | 0.789 | 2.01 | 39.2 |
| 3 | WPL 10% | 0.077 | 1.51 | 13.38 | 13.9 |
| 4 | Casein 5% | 0.229 | 2.48 | 3.15 | 7.8 |
| 5 | Casein7.5% | 0.122 | 0.4 | 442.45 | 121.1 |
| 6 | Casein 10% | 0.396 | 0.129 | 83.5 | 50.4 |

The findings in Table (4-1) reveal that the protein type and the protein level had a strong influence on the physical and mechanical properties of the edible films. Casein and whey protein films behaved differently and this showed that there was a difference in the molecular structure, network formation and protein-protein interactions.

The thickness of the film was not linearly proportional to the concentration of the protein used. The films of whey protein prepared at the level of 10% demonstrated the lowest value of thickness (0.077 mm), which can be explained by the partial precipitation of the protein or unequal dispersal of the protein in the course of the casting procedure. This implies that the thickness of the film is more influenced by solubility of the protein and stability of the network than concentration of the protein. The same was noted by [32,33,34], who pointed out the importance of molecular interactions in the film structure determination. The statistical examination revealed that there are significant differences in the formulation thickness ($p < 0.05$).

Protein concentration also had a great impact on tensile strength. The tensile strength peaked at 7.5% concentration in the case of whey protein films, which demonstrated a better intermolecular bonding as well as network cohesion. Nevertheless, it was found that there was a significant decrease at 10 percent concentration of both whey protein and casein films ($p < 0.05$). This decrease can be due to too much protein aggregation that interferes with the even formation of networks giving rise to brittle structures. This was also observed by [32,35] who noted that at high concentrations of protein or plasticizers mechanical weakening occurred.

The values of modulus of protein types were different in Young. The modulus value of whey protein films was relatively low indicating that it is very flexible and less rigid. Conversely, casein films with 7.5% exhibited an exceptionally high Young modulus (442.45 Mpa), which revealed that it possessed a stiff and rigid matrix. These actions could be connected with the high interactions of casein-casein and thick protein packing. According to[36], overloading of casein may destroy the cohesiveness of the film even when it becomes stiffened. There was statistical significance ($p < 0.05$) between the differences in the observed modulus of Young.

Break elongation was significantly different across treatments. At 7.5% the highest frequency of elongation (121.1% was obtained) was obtained though accompanied by very large modulus which is indicative of a stiff but stretchable structure. Conversely, the elongation values of whey protein films were more balanced, meaning that it is well-constrained in terms of both flexibility and mechanical stability. Such findings are consistent with [35] who reported moderate protein concentrations increase the extensibility, whereas higher protein concentrations favor aggregation and decrease flexibility. There was statistically significant difference in the elongation under treatments ($p < 0.05$).

All in all, the data shows that there is a high interdependence of thickness, tensile strength, Youngs modulus, and break elongation. Movies that were made at protein concentration of 7.5% had a better internal bonding that led to increased tensile strength and extensibility than low and high concentrations ($p < 0.05$). On the other hand, movies made at the concentration level of 10 percent especially whey protein films displayed a reduced thickness and poor mechanical performance. The findings are in line with that of[32,35].

According to [37], the protein concentration of 7.5% particularly with whey proteins that were

plasticised with glycerol and added with probiotics under neutral pH conditions yielded the most balanced mechanic strength, flexibility and structural integrity. The statistically significant differences ($p < 0.05$), along with the results of microbial stability, prove the choice of this formulation as a promising candidate of functional probiotic-enriched edible films.

**Table (4-2) Mechanical properties of manufactured edible protein films supported by probiotics**

| Proten and probiotic | Thickness (mm) | Tensila Stress (Mpa) | Young s Modulus (MPa) | Elongation at break% |
|---|---|---|---|---|
| **Wpl 7.5% , *Bifidobacteria*** | 0.232 | 57.91 | 470.0 | 171.0 |
| **Wpl 7.5% , *Saccharomyces boulardii*** | 0.552 | 6.55 | 4.78 | 137.5 |
| **Casein 7.5%,*Bifidobacteria*** | 0.246 | 63.96 | 653.0 | 137.2 |
| **Casein 7.5%, *Saccharomyces boulardii*** | 0.310 | 72.78 | 2480.0 | 146.0 |

Table (4-2) of the mechanical properties of probiotic-supported edible protein films reveals that the incorporation of probiotics had a major effect on the film structure and performance, which depended on the type of protein and the strain of probiotic. The tensile strength, Youngs modulus, and the elongation at break were statistically different between probiotic containing films with the addition of both Bifidobacterium and Saccharomyces boulardii ($p < 0.05$).

Whey protein films strengthened using Bifidobacterium had an enhanced mechanical performance with a tensile strength of 57.91 Mpa and an elongation of the break amounting to 171%. This is a significant improvement in relation to the similar whey protein films that lacked the probiotics and were found to be of less extensibility and strength. The positive interactions between bacterial cells and whey protein chains may be considered the cause of these improvements that contribute to a stronger intermolecular bonding and higher network continuity. This has been observed in other studies such as [38] who established that probiotic bacteria can serve as active fillers in protein-based matrices.

Conversely, whey protein films infused with Saccharomyces boulardii exhibited significant enhancement of elongation at break up to 137.5% with a significant drop in tensile strength and Youngs modulus ($p < 0.05$). This tendency implies that incorporation of yeast promotes film flexibility, as well as decreases rigidity and stress resistance. The size and other surface properties of yeast cells are relatively larger and this could cause some disruption of protein-protein interactions and form less uniform networks and results in films which are softer and more elastic. Similar effects were characterized by [39], which stated that cohesive protein linkages can be weakened by addition of yeast, and extensibility enhanced.

Films made of casein showed a striking reaction with interventions of probiotics. The tensile strength and modulus values were better in films supplemented with Bifidobacterium, which suggests the creation of a dense and mechanoresilient matrix. On the other hand, the films with Saccharomyces boulardii were characterized when compared to their counterparts by an increase in elongation at break, to a maximum of 146% ($p < 0.05$), at the cost of tensile strength and stiffness. The results are in line with results of [40], who reported that incorporation of bacteria into sodium casein films enhanced strength and extensibility and minimally altered Youngs modulus. This supports the idea that bacterial probiotics will be associated with cohesive network formation and that the primary role of the yeast cells is to be associated with flexibility.

On the whole, statistical data supported the finding that the most balanced mechanical behavior of whey protein and casein matrices was observed in films with 7.5% protein concentration and with no differences on the type of probiotic ($p < 0.05$). Microbial stability

tests also supported this formulation as optimal giving it superior mechanical performance and probiotic viability. These findings confirm that probiotic addition does not only alter mechanical properties but also adds to the functional and bioactive capacities of edible films, which justifies their appropriateness in eco-friendly and biodegradable packaging of foods [41]. Further hydrogen bonding and strong interactions between bacterial cells and protein chains enhance the strength of tensile strength and elongation of Bifidobacterium reinforced films, which result in better network cohesion. Films containing Saccharomyces boulardii show increased flexibility and elongation because yeast cells disrupt uniform protein interactions, creating a softer and more elastic network.

### *4.1 Characterization of the prepared flims*

#### *4.1.1 Fourier transform infrared spectroscopy (FTIR)*

Figures (4-1) and (4-2) indicate FTIr spectrums of the prepared protein-based membranes in the wavenumber of 400-4000 cm-1, and Table (4-3) is a summary of the typical vibrational bands related to the primary functional groups. The spectra of all the membranes had a broad absorption band at around 3401-3434 cm-1 that is attributed to the overlapping n(OH) and the n(NH) stretching vibrations of hydroxyl and amino groups respectively. This large band indicates that there are a lot of hydrogen bonding reactions in the protein matrix especially in using OH groups as well as NH2 groups that are natural in milk-derived proteins. Hydrogen bonding such as this is important in the stabilization of the film structure by increasing the level of intermolecular cohesion and network formation. The same spectral characteristics have been observed [42], who attributed this band to strong hydrogen-bonded interactions with in protein-based films. The fact and strength of this band suggest that protein chains are well crosslinked with each other which explains the mechanical stability and elasticity of the membranes.

**Table (4-3) Diagnostic vibration table for membranes prepared from casein and whey in infrared spectroscopy**

| *Assignment group* | *Wavenumber ($cm^{-1}$)* | | | | | |
|---|---|---|---|---|---|---|
| | **Casien 5%** | **Casien 7.5%** | **Casien 10 %** | **WPL 5%** | **WPL 7.5%** | **WPL 10%** |
| **ν(O-H, N-H) overlapping** | 3434 | 3401 | 3411 | 3434 | 3430 | 3430 |
| **ν C-H (-$CH_3$)** | 2924 | 2925 | 2925 | 2924 | 2924 | 2924 |
| **ν C-H (-$CH_2$)** | 2854 | 2854 | 2854 | 2853 | 2854 | 2854 |
| **δ(-CO-NH) primary amide** | 1631 | 1642 | 1633 | 1632 | 1632 | 1632 |
| **δ(-CO-NH) secondary amide** | 1458 | 1456 | 1457 | 1465 | 1464 | 1465 |
| **δ C-H ($CH_2NH_2$)** | 1385 | 1385 | 1385 | 1384 | 1384 | 1385 |
| **δ C-H** | 1270 | 1262 | 1262 | 1271 | 1266 | 1270 |
| **ν (C-O-C)** | 1155 | 1114 | 1114 | 1114 | 1113 | 1113 |
| **δ (C-O) in (C-OH)** | 1047 | 1040 | 1040 | 1040 | 1045 | 1048 |

The FTIR spectra of whey protein and casein membranes were used to determine the diagnostic vibrations of these proteins, as shown in Table (4-3). The amide I region (1642 cm-1), which is linked to C=O stretching vibrations, the amide II region (1458 cm-1), linked to N-H bending in N-C=O groups, and the amide III region (1270 cm-1) linked to C-N stretching of peptide bonds were typical of all membranes [43,44]. These peaks make sure that the specific protein structures and peptide connections are intact, which are the keys to forming the film and ensuring its mechanical stability. Moreover, the presence of peaks between 1160-720 cm-1 means that the membranes have important diagnostic vibrations [45]. Particularly, the highest peak at 1114 cm-1 is due to the stretching vibration of

the glycosidic C-O-C bond of lactose, an increase in the value of which and a shift in wavenumbers with the rising protein concentration. The highest peak in 1040 cm-1 color is due to C-O stretching in alcoholic groups of lactose and glycerol and the highest peak in 720 cm-1 is due to bending vibrations of the glycosidic bonds [46]. A combination of these spectral characteristics reveals that the protein matrix contains powerful interactions involving hydrogen bonding and the creation of peptide networks, which are factors that act towards the observed mechanical properties, stability, and tendencies of the edible films.

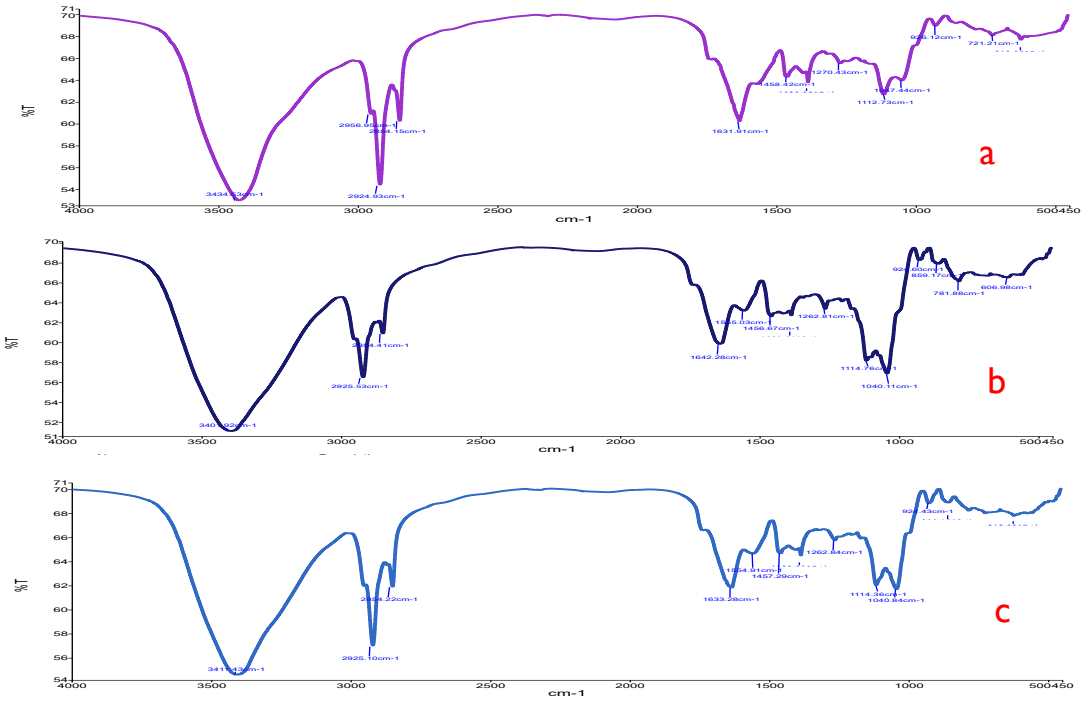



**Figure (4-1): Infrared spectrum of membranes prepared from casein (a 5% (b) 7.5% (c) 10%**

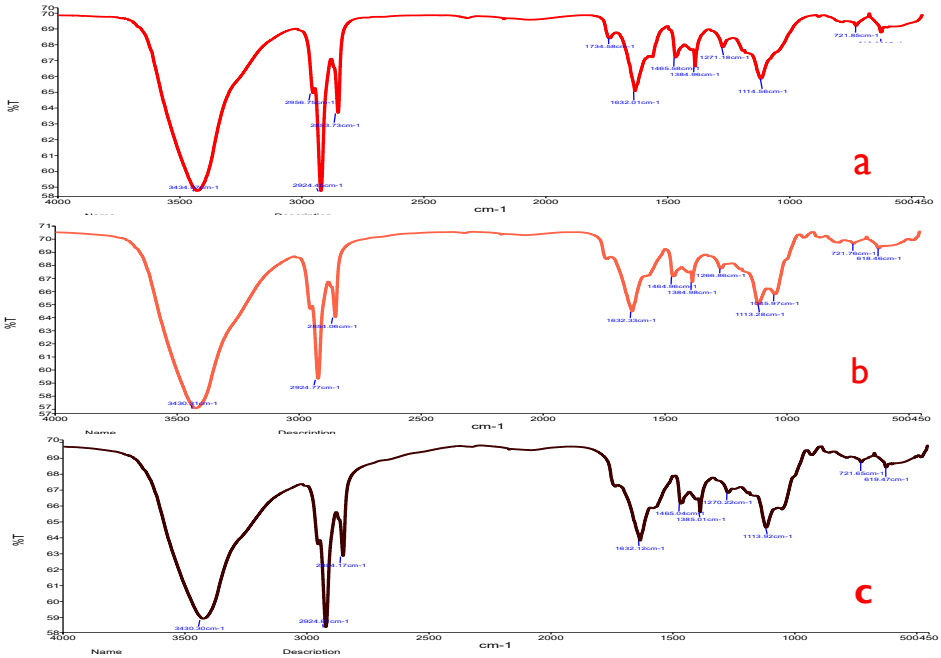



**Figure (4-2): Infrared spectrum of membranes prepared from whey (a) 5% (b) 7.5% (c) 10%**

The presence of characteristic spectrum peaks in the FTIR analysis of protein membrane reinforced with bacteria and yeast was indicative of the interactions that the biostimulant exhibited with the constituents of the membrane structure.

**Table (4-4) Diagnostic vibration table of membranes prepared from casein and whey, supplemented with probiotics in the infrared electromagnetic spectrum.**

| | Wavenumber ($cm^{-1}$) | Assignment Group |
|---|---|---|
| WPL + *Bifidobacterium* | 3432 | ν(O–H, N–H) overlapping |
| | 1662 | δ(–CO–NH), Amide I (primary amide) |
| | 572–518 | S–S / C–S: sulfur bonding |
| WPL +*Saccharomyces boulardii* | 2127 | C≡N / $CO_2$: secondary degradation or noise |
| | 1385 | δ(C–H, $–CH_2NH_2$) |
| | 484–431 | Calcium–protein interactions |
| Casein +*Saccharomyces boulardii* | 2921 | ν(C–H, $–CH_3$) |
| | 970.02 | P–O–C: phosphate |
| | 663–534 | Modified sulfur bonds |
| Casein + *Bifidobacterium* | 848.52 | P–O stretching: exopolysaccharide |
| | 696–628 | C–S / tryptophan ring |

Table (4-4) will show the diagnostic vibrations of whey protein and casein membranes with probiotics. The large peak of 3432.67 cm-1 in whey protein films strengthened with Bifidobacterium is associated with overlapping n(O-H) and n(N-H) vibrations and represents the significant amount of hydrogen bonding and the interaction between the protein chains and bacterial polysaccharides [47]. The range of 572-518 cm-1 indicates that there are peaks which indicate increased sulfur bonding (S-S, C-S) that leads to the mechanical stability improvement [48]. The center of the Amide I region at 1662.34 cm-1 is a surety that the secondary a-helix structure of proteins does not change significantly, but rather there are minimal changes that bacterial enzymes cause .[٤٩]

The maximum at 2921.63 cm-1 indicates hydrophobic interactions between yeast b-glucans and protein side chains in casein films supplemented with Saccharomyces boulardii

[50] although the appearance of a peak at 970.02 cm-1 indicates the formation of more phosphate bonds and this may have been as a result of yeast phosphatase activity [51]. The 663-534 cm -1 peaks suggest changes in sulfur bonds, which is in line with the enzymatic impacts on sulfur-containing amino acids cysteine and methionine [52]. A peak of 2127.1 cm-1 also appeared in whey protein films reinforced with yeast, and could either be due to secondary pronuclei of the degraded protein, or could be due to overlapping C[?]N/CO2 signals [47]. Moreover, the peaks of 484- 431 cm -1 indicate calcium-protein interactions that amplify cross-linking of the protein and the entire mechanical characteristics.[٥١]

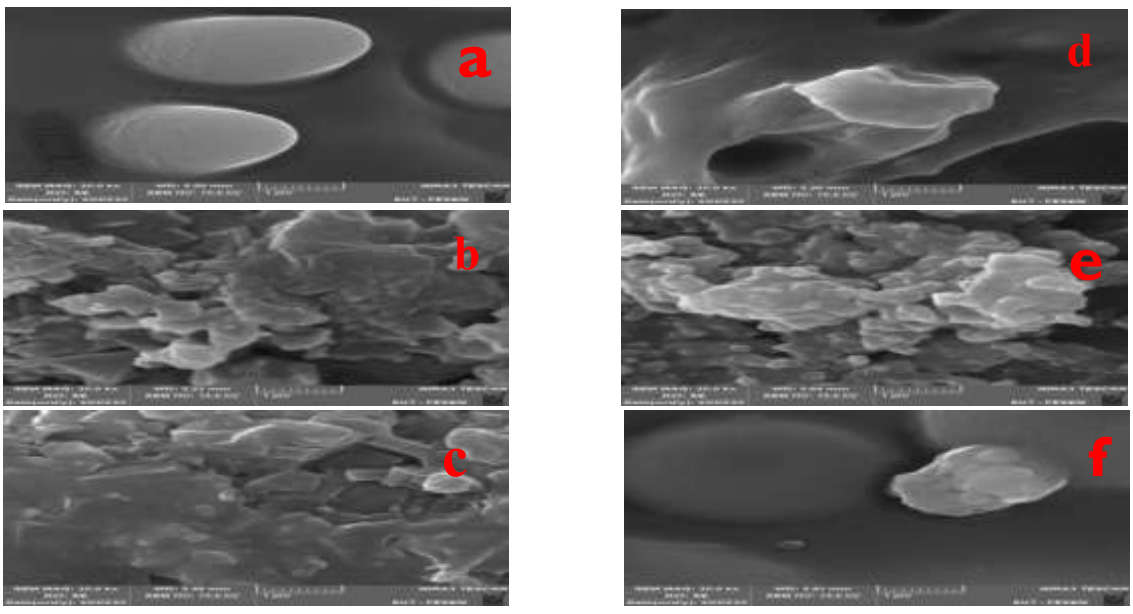
These spectral observations demonstrate that addition of probiotics to protein-based films not only maintains the protein secondary structure, but also causes other degrees of molecular interaction e.g. hydrogen bonding, sulfur bond alteration, phosphate bond, and hydrophobic interactions. It is expected that such interactions are the basis of the recorded tensile strength, elasticity, and flexibility enhancement in probiotic-enriched edible films, which makes them a promising functional and bioactive packaging material. These FTIR variations prove that probiotic additions alter the interactions between the moleculars in the protein matrix. Bifidobacterium enhances the hydrogen bonding and strengthens the network increasing the mechanical stability, whereas Saccharomyces boulardii alters the phosphate and sulfur bonding, increasing the flexibility and elongation.

***4.1.2 Scanning electron microscopy (SEM)***

Figure (4-3) shows SEM pictures of whey protein and casein membranes made at various concentration with apparent differences in the surface morphology and porosity. Membranes loaded with 5% casein or whey (Figure 4-3a and 4-3d) had comparatively smooth surfaces whereby the casein films had spherical protrusions and the whey films had small pores. Having pores enhances the surface area and could support the growth of microorganisms, but smooth surfaces are desired in processes that need less contamination [53].

The rough and porous textures of membranes made at 7.5 percent concentration of protein (Figure 4-3b and 4-3e) had visible agglomeration of membrane particles on both surfaces. This coarse and scaffolded morphology enhances the effective surface area of the films, which may confer mechanical interlocking and could be used to improve probiotic incorporation [54,55].

Membranes prepared using 10% protein (Figure 4-3c and 4-3f) on the other hand had a smoother, non-porous surface with less agglomeration of particles than the 7.5% films. These properties of the surface are desirable to reduce microbial colonization and contaminant retention, enhance film stability and functionality in processes with strict selectivity and biofouling resistance requirements. These findings of the SEM are associated with mechanical and microbial stability data, indicating that the concentration of proteins and the morphology of the surface are important factors in identifying functional characteristics of probiotic-enriched edible films.



**(4-3)SEM images of the membranes prepared with different ratios of casein (a) 5% (b) 7.5% (c) 10% and whey (d) 5% (e) 7.5 %(f) 10%**

Figure (4-4) shows SEM photos of biopolymer membrane that has been prepared with casein concentration of 7.5% (whey) and modified with probiotic microorganisms (Saccharomyces boulardii or Bifidobacterium). That casein membrane strengthened with S. boulardii (Figure 4-4a) had an irregular and heterogeneous surface with irregularities and could have micro-porosity. These characteristics would be formed probably due to aggregation of the yeast cells partially or not, or uneven dispersion of the yeast cells in the protein matrix which may affect both the permeability and the mechanical behavior of the membrane [56].

However, the Bifidobacterium reinforced casein membrane (Figure 4-4b) showed a smoother and more homogenous architecture. This even distribution of cells in the bacteria seems to lead to a more condensed and stable membrane with better structural integrity and less void formation [57].

The S. boulardii whey membrane (Figure 4-4c) had a rather porous and irregular surface. This porosity can be explained by the reduced weight of molecular fractions and unique architectural features of whey proteins in comparison with casein along with the presence of yeast. Although this type of structure can be advantageous to functional characteristics such as controlled release, it might weaken mechanical properties [58].

On the other side, the bacterial inclusion in the whey membrane reinforced with Bifidobacterium (Figure 4-4d) had a more smooth and dense surface, which demonstrates that the incorporation of bacteria improves the microstructural homogeneity and cohesion. In general, the analysis of SMEs supports the idea that bacterial reinforcement characteristically forms more homogenous, dense, and mechanically resistant membranes, whereas the incorporation of yeast cells is likely to cause irregularities and porosity on the surface of the structure, lowering its structural homogeneity and robustness [59]. All these SEM observations suggest that incorporation of bacteria results in smoother, denser, and more cohesive surfaces which are correlated to increased tensile strength and mechanical integrity. Integration of yeast produces irregular porous surface which justifies the rise in flexibility and elongation compared to the reduced rigidity. These descriptions are corroborated by the mechanical property data and it is possible to conclude that bacterial-enriched films are superior to yeast-enriched films in terms of the surface morphology and mechanical integrity.

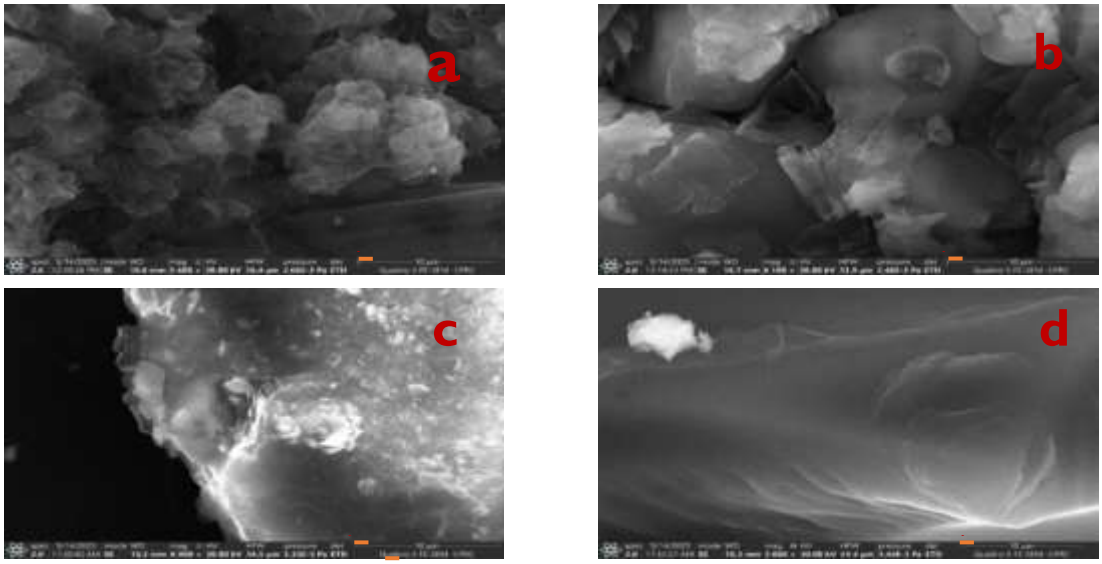



Figure (4-4): SEM images of the prepared membranes (a) 7.5% casein enhanced with yeast (Saccharomyces boulardii)(b)7.5% casein enriched with bacteria (Bifidobacterium) (c) 7.5% of WPL with yeast (Saccharomyces boulardii ) (d) 7.5% of the mash and enriched with bacteria (Bifidobacterium).

***3- Energy-dispersive X-ray spectroscopy (EDX)***

SEM was used together with energy-dispersive X-ray spectrophotometry (EDX) to examine the elemental structure of the protein-based films. Figures (4-5) and (4-6) represent EDX spectra of membranes made of different amounts of whey and casein. The results of the spectrums showed that there were carbon (C), oxygen (O), nitrogen (N), phosphorus (P), sodium (Na), calcium (Ca), magnesium (Mg), and sulfur (S in different proportions) and hydrogen was absent because of the limitations of the EDX technique. The elemental ratios were found to be different indicating the differences in elemental protein type, concentration, and possible surface changes.

The occurrence of these elements qualitatively certifies the chemical contents of the membranes as opposed to the actual quantitative data. Aesthetic peaks can also be associated with surface oxidation, trace contamination, or adsorbed material at the nanometer level, which are difficult to measure due to the fact that EDX measurement can be created by both the surface and subsurface layers, and may average local surface effects [60].

Table (4-7) and (4-8) show the EDX spectra of supplemented membranes with probiotics. They were found to exhibit similar elemental profiles and this showed that the incorporation of probiotics did not bring new elemental species, but could affect the distribution and relative strength of the elements that were already present to the system, which was probably because of the interactions between chains of proteins and microbial components. The EDX is a stable qualitative approach in the identification of characteristic elements, but quantitative analysis in complicated biological systems should be meticulously calibrated because of the likely overlapp of spectral peaks and effects of the matrix [61]. The elemental structure remains largely the same, however, owing to the change in element distribution in probiotic-supplemented films, microbial addition seems to alter protein network organization, which subsequently impacts mechanical and functional performance.

Altogether, EDX analysis sustains compositional consistency of the films and ensures the existence of the major protein and mineral-associated factors to generate the background information to explain the mechanical, structural, and functional habits of both non-improved and probiotic-added protein membranes.

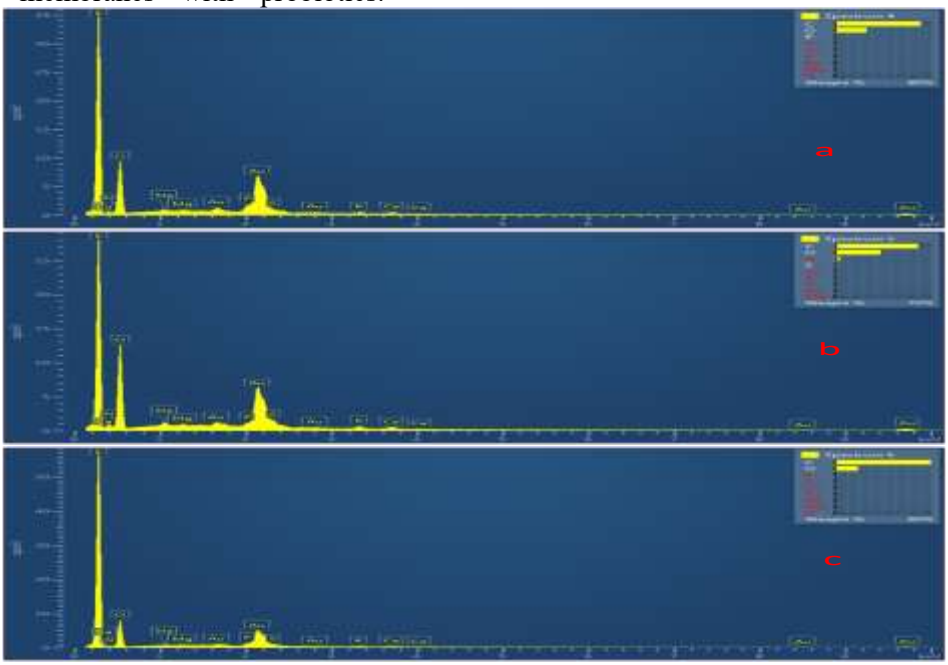



**Figure (4-5): EDX spectrum of membranes prepared with different whey concentrations (a) 5% (b) 7.5% (c) 10%**

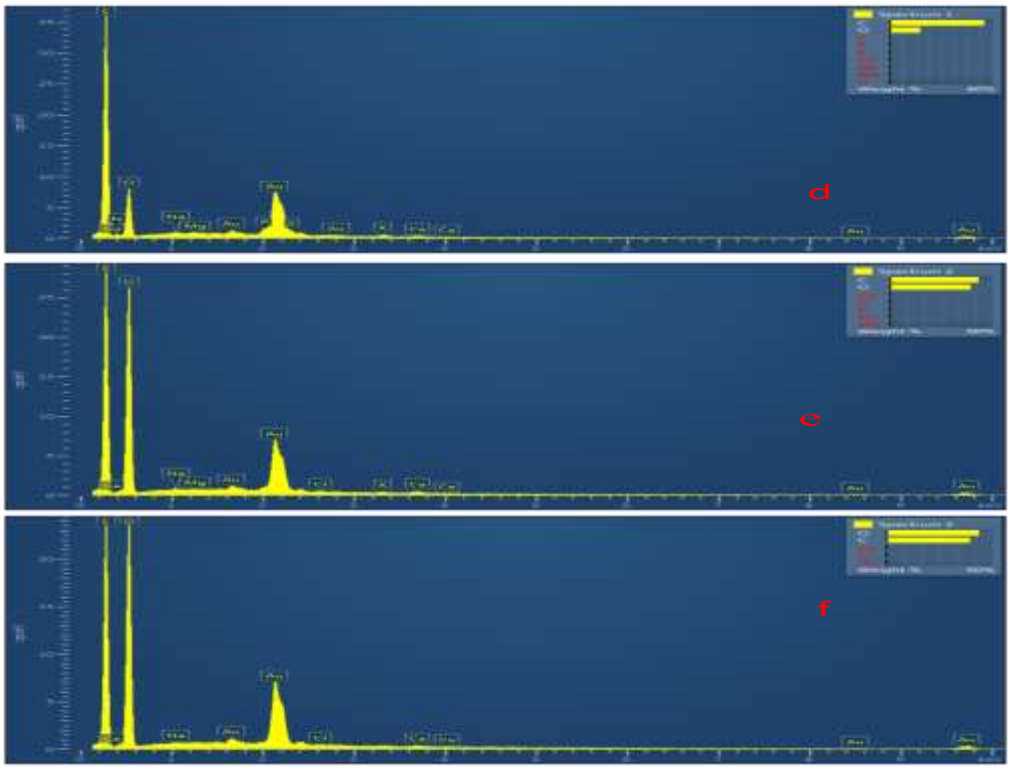



**Figure (4-6): EDX spectrum of membranes prepared with different ratios of casein (d) 5% (e) 7.5% (f) 10%**

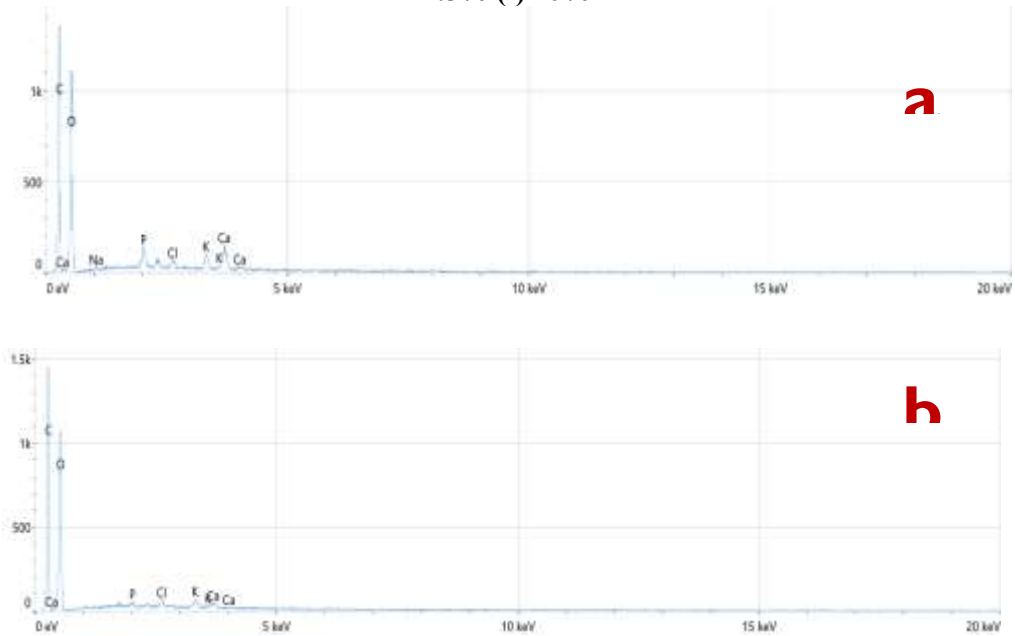



**Figure (3-7): EDX spectrum of the prepared membranes Figure (4-7)(a) 7.5% casein enhanced with yeast (*Saccharomyces boulardii* ) (b) 7.5% casein enhanced with bacteria (*Bifidobacterium*)**

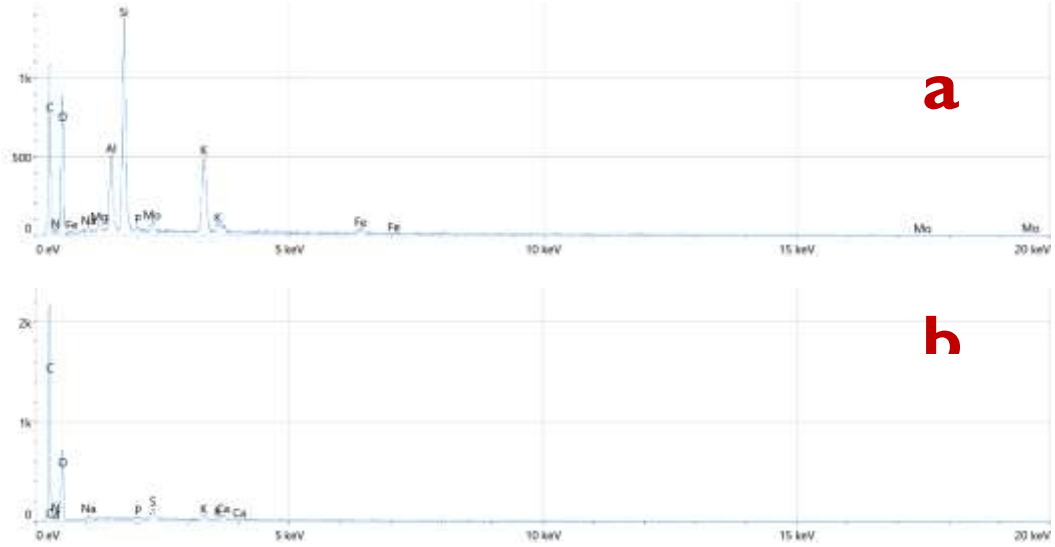



**Figure (4-8): EDX spectrum of the prepared membranes (a) 7.5% of the WPL enhanced with yeast (*Saccharomyces boulardii)* (b) 7.5% of the substrate enhanced with bacteria (*Bafidobacterium*)**

## 5- Conclusion

In this study, edible protein-based films were successfully developed using whey protein and casein, supplemented with probiotic strains, Saccharomyces boulardii and Bifidobacterium. Structural, mechanical, and functional analyses demonstrated that a protein concentration of 7.5% provided the most balanced properties, including optimal tensile strength, elongation, surface homogeneity, and microbial stability. The incorporation of probiotics influenced film characteristics differently: bacterial enrichment (Bifidobacterium) enhanced mechanical integrity and produced smoother, denser surfaces, whereas yeast (Saccharomyces boulardii) mainly increased film flexibility and porosity, consistent with SEM, EDX, and FTIR analyses.

These movies present a promising approach to probiotic delivery during the functional food packaging as they are able to protect and release viable microorganisms in a controlled way. Their application would give nutritional and health value to the food products and help to enable the creation of biodegradable and eco-friendly packaging materials.

These films have potential in achieving sustainable packaging despite having some drawbacks of conventional plastics such as sensitivity to moisture and mechanical strength. The next steps towards formulation optimization, enhancement of barrier properties, and scalability across different food matrices should be recommended to obtain functional, industrially viable and safe packaging solutions.

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### Conflict of interest

The authors have no conflicts of interest to disclose that are compatible with the subject matter of this article.

### Consent to participate

All authors have expressed their authorization to engage in this publication.

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### Ethical Statement

Except for sensory evaluation, we have not conducted any human or animal investigations during this study. Regarding the sensory case.

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