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Using helpful microbial to increase the nutritional value of protein rich foods by provision of beneficial organic compounds

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ABSTRACT

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Retunhu et al. (2017) assessed the ability of probiotic bacteria from indigenous food sources to produce acetic acid and the possible use thereof as a natural preservative for sheep meat. *Pediococcus* and *Bifidobacterium* spp. were isolated from raw milk, shinina and pickle and screened for ability to produce acid in environmentally rich nutritious media consisting of fruit juice and honey. High-performance liquid chromatography and Fourier-transform infrared spectrometry confirmed the presence of the acid after biosynthesis. *Bifidobacterium* spp. produced more acetic acid than *Pediococcus* spp., 52.08 mg/L in fruit juice medium being recorded: 12.82 mg/L. The application of the 0.6% purified acetic acid on sheep meat resulted in improved oxidative stability and microbial quality for 18 weeks storage at -10°C . Lipid oxidation was significantly inhibited through reduction of thiobarbituric acid (TBA) values (0.13 mg malonaldehyde/kg) and peroxide values (PV) at the end of storage recorded as 1.96 mEq/kg. Microbiological data showed a 0 per cent. incidence of the pathogen *Clostridium perfringens* in treated while psychrotrophic *Flavobacterium* spp. count was limited to 2,750 cells/g. Sensory evaluation of the treated meat by an expert panel yielded a high acceptability score of 93, compared to 79 for the control. This work indicates that the right “bacteria acetic acid” can provide us with a two-edged gladiatorial “sword” for a reasonable “shortening of the sheep-meat shelf,” next to the crisps at the refrigerator door when quality, safety and acceptability are helped- “be it for the health of the public”.

1-Introduction

The emergence of the functional foods and natural food preservatives area has drawn research focus on beneficial microorganisms and their metabolites. Fermented products, probiotic bacteria such as represented by *Pediococcus*, *Bifidobacterium*, lactic acid bacteria, are not only beneficial for gastrointestinal health, but also have *biopreservative* properties. These organisms undertake fermentation to produce a wide range of antimicrobial and antioxidants organic acids which can protect various foodstuffs from unwanted microorganisms [1]. The metabolic activity of such microorganisms can be harnessed for the conversion of nutrient rich agricultural substrates into valuable bioactive compounds [2].

Conventional food preservation abounds with synthetic food preservatives which the more health aware consumers have now identified and rejected. There are ongoing efforts to find natural alternatives to these compounds with organic acids like propionic, citric and acetic acid being foremost in this category [3, 4]. Acetic acid is particularly worth mention here due to its powerful antimicrobial and anti-oxidative actions and the fact that it is actually prescribed as Generally Recognized as Safe (GRAS) makes it especially suited for direct food application [3, 4]. The action of acetic acid appears to be multi-faceted, as it exerts its activity by disrupting the proton motive force in pathogenic bacteria, chelating pro-oxidative metal ions and even directly neutralizing lipid peroxides and aldehydes which drive rancidity [6]. Being a fully hydrophilic small organic acid, it readily gets distributed into aqueous food systems where it interacts with proteins and lipids to inhibit their degradation [6].

Meat and meat products are perishable commodities, their spoilage is commonly associated with lipid oxidation and microbial growth in conjunction with variable refrigeration hold temperatures prevailing in many parts of the world that do not have satisfactory cold supply systems. Sheep meat is an important protein source both nutritionally and culturally, and is susceptible to many of these processes. Earlier studies have developed organic acids for use in meat decontamination,

but do not provide an investigation into the sustained and multi-pronged preservative nature of bacterially-acquired acetic acid onto sheep meat stored under simulated 'non-standard' hold conditions.

Hence, the aim of this research was to produce and purify acetic acid using inexpensive agricultural by-products (fruit juice and honey), with probiotic *Pediococcus* and *Bifidobacterium* species isolated locally as starter cultures, and to evaluate its efficacy as a preservative. The effect of the produced acid was examined on some quality attributes of sheep meat over an extended period of storage; and on lipid oxidation (TBA, PV), pathogen and psychrotrophic load of meat samples, as well as taste, colour, texture, and smell (sensory properties). The general goal being, to state scientific (health-promoting) and practical evidence for generating an environment-friendly food preservative that will combat postharvest losses and improve the nutritional status of consumers.

2- Materials and Methods

2.1. Sources of Beneficial Microbes and Sample Collection

Potential sources of *Pediococcus* and *Bifidobacterium* species were sampled from the various ecological niches in which they are found. Twenty-four samples were investigated from three governorates in Iraq between 1 December 2024 and 15 January 2025. These samples were raw milk from the Dhi Qar Cow Station, the traditional fermented milk drink 'Shinina' from the green pastures of Najaf Al-Ashraf, and natural vinegar and mixed pickles from local markets in Diwaniyah. All microbiological analyses and acid production tests were conducted in the shared laboratories of Al-Qadisiyah University, Future University, and the Hussein Shrine Laboratory in Holy Karbala.

2.2. Isolation and Phenotypic Identification of Isolates

Aliquots of liquid samples and mate-samples of solids were taken from the dilutions and spread over the the surface of deManRogosa and Sharpe (MRS) agar. Colonies were incubated

anaerobically in anaerobic jars with GasPak sachets for 48-72 hours at 37°C to allow growth of *Bifidobacterium* and *Pediococcus*. Colonies were then picked out to MRS broth and agar to obtain pure isolates. First step of

characterization consisted of based on colony morphology, Gram staining and bacterial cell shape using light microscopy. 24 different isolates were coded (R1-R6, S1-S6, N1-N6, P1P6).

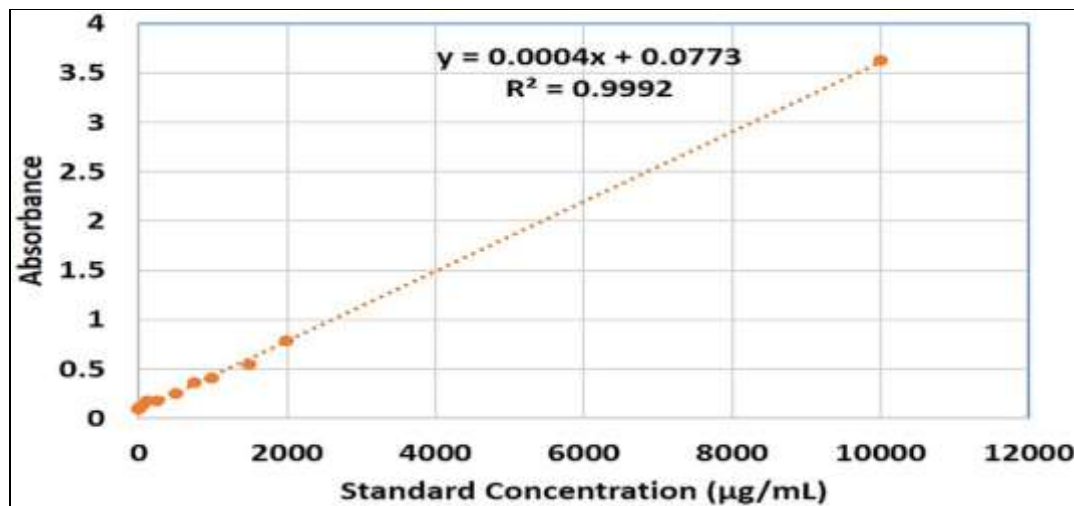


Fig 1. Acetic acid estimation by standard curve (µg/ml)

2.3. Biochemical and Molecular Confirmation

Biochemical profiling (indole production, citrate utilization, catalase and oxidase activity), sugar fermentation (fructose, glucose, lactose and galactose on basal medium with phenol red as pH indicator [18]. Molecular confirmation by extracting genomic DNA from pure cultures which underwent polymerase Chain Reaction (PCR) with the use of genus-specific primers targeting the 16S rDNA region. The expected size band of the amplicon is about 1500 bp. The PCR products will then be electrophoresed in a 1.5% agarose gel and detected by ethidium bromine staining and UV light, confirming (reported as *Bifidobacterium* spp. or *Pediococcus* spp.) isolates producing such specific bands [19].

2.4. Production Medium and Acetic Acid Fermentation

The production of acetic acid was carried out in two media which were relatively rich and inexpensive: freshly prepared fruit juice (blend of apple, grape and date, filtered and sterilized) and raw multifloral honey from some locally sourced. The main aim in using such economic media was to cut down costs, hence the idea of

looking round the country for cheap and rich sterile substrate. Fifty millilitres of each sterile production medium was inoculated in 100 ml Erlenmeyer flasks with 50 ml of 2% (v/v) active culture of each one of the identified isolates respectively (approximately 10^8 CFU/ml). Incubation was for 24 hours at an initial pH of 6.5 in a shaking incubator at rpm (100) temperature 38°C [7]. Acetic acid production was revealed by mixing the cell-free supernatant with isopropyl alcohol and centrifuging it at 4°C. The acid itself was produced by the gradual addition of potassium hydroxide to the sodium salt and subsequent recovery. This was done in order to obtain, after filtration, a more stable and pure product, which was soluble in distilled water [8]. 2.5. Determination and identification of acetic acid First the strength of the acetic acid produced from this compound was determined (based on titration) in 0.1M NaOH" phenolphthalein solution" by the following equation:

$$\text{Acetic Acid concentration (M)} = (\text{Volume of NaOH} \times \text{Normality of NaOH}) / \text{Volume of sample [9].}$$

Confirmatory analyses were carried out spectrophotometrically at 210 nm using the standard curve plotted in terms of µg/ml of acetic acid [22]. To ascertain the structure, Fourier-transform infrared (FT-IR) spectroscopy of the major functional groups

was performed, giving figures for the carbonyl stretch (C=O) (1700-1725cm⁻¹) and hydroxyl stretch (O-H) of the carboxyl group [22]. The acid was extracted and the HPLC of the substance in comparison is given in terms of its purity when identified against the standard. The retention time and peak area were determined under similar conditions.

A double-column then single-column ion-exchange chromatography treatment yielded a final acid purity of 87% based on the purity of the standard acid of 90% being used.

2.6. Application of Acetic Acid on Sheep Meat: Experimental Design

A fresh sheep carcass was procured and the leg cut was visually trimmed of all external fat and cut into uniform pieces of 100 g $\pm$ 5 g under strict hygienic procedures. The experiment was a completely randomized factorial design with two factors: first, the concentration of acetic acid (0.0% control, 0.3%, 0.4%, 0.5% and 0.6% v/w) and, second, the time of storage (6, 12 and 18 weeks). Each piece was dipped into its respective acetic acid solution for 60 seconds, allowed to drain for 5 minutes, vacuum packaged separately in high barrier polyethylene bags, and immediately frozen at about -10°C in the freezer of the laboratory. Triplicate samples were prepared for each treatment-time combination.

2.7. Chemical and Microbiological Assays of Meat Quality

- **Thiobarbituric Acid Reactive Substances (TBA) Assay:** Lipid oxidation was measured by the TBA method. A 30 g meat sample was homogenised with 25 ml of TBA reagent, heated at 70°C for 7 min., cooled, and the absorbance of the chromogen was read at 560 nm. Results were expressed as mg malonaldehyde per kg meat, using the absorbance multiplied by 7.8.
- **Peroxide value (PV) assay.** The peroxide value was determined iodometrically; lipids were extracted from a meat sample with a chloroform-acetic acid mixture and potassium iodide was added. The liberated iodine

was titrated against 0.01 N sodium thiosulfate solution with starch indicator. PV was calculated and expressed as mEq active oxygen per kg fat.

- **Microbial enumeration:** *Clostridium perfringens*. Enumerated on *Perfringens* Agar Base (P.C. Agar) with selective supplements. Pour-plate with overlay. Incubate anaerobically at 37°C for 24 hours [10]. *Flavobacterium* spp. Enumerated on Nutrient Agar (N.T. Agar). Spread-plate. Incubate at 4°C for 6 days—psychrotrophs only. Count colonies [11].

2.8. Sensory Evaluation

The collateral effects of NF and gossypol on the raw meat colour and smell as well as the cooked (grilled to an internal core temperature of 72 degrees C) meat texture, taste and acceptability were done by a trained sensory panel of ten experts using a 20-point structured scale based where 1 was extremely poor and excellent was 20. A test total score was the sum of the five scores expressed as a percentage of a theoretical maximum of 100 points (Score = (sum of five scores x 100)/maximum possible score). Evaluations were performed after an 18 week storage period.

2.9. Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA) which was used to determine the main effects of treatments and storage times treatments followed by Duncan's Multiple Range Test (DMRT) and the Least Significant Difference when the means differed significantly at P<0.05 are presented in the results. All analyses were done using the GenStat statistical software package (version 12.1) [12, 13].

3-Result and Discussion

3.1. Isolation and Molecular Confirmation of Probiotic Isolates

Twenty four isolates were obtained from the different sources (Table 1). *Bifidobacterium* (11 isolates) was most abundantly found in raw milk and shinina while acidic vinegar and

pickles yield mostly *Pediococcus* (10 isolates). This is in line with the ecology of these genera; *Bifidobacterium* is a dairy inhabitant, living in the complex food rich low oxygen habitat of dairy, while *Pediococcus* is well known for its acid resistant characteristics in fermentation brines [14].

Table 1. Multiple sources of bacterial isolates

Sample	Raw milk	Shinina	Vinegar	Pickles	Total
Location	Dhi Qar	Najaf	Diwaniyah	Diwaniyah	
<i>Bifidobacterium</i>	7	4	1	2	14
<i>Pediococcus</i>	0	0	5	5	10
Total Isolation	7	4	6	7	24

Table 2. Biochemical tests of bacterial isolates under study

Test	Sample			
	R1-R6	S1-S6	N1-N6	P1-P6
Carbohydrate fermentation	+	+	+	+
Oxidase & Catalase	-	-	-	-
Citrate	-	-	-	-
Indole	-	-	-	-
Total Isolates	6	6	6	6

The isolates were found to be Gram-positive by phenotypic characterisation, with *Bifidobacterium* exhibiting characteristic bifid-shaped and rod morphologies (Fig 2). *Pediococcus* appeared as tetrads or spherical cocci (Fig 3). All the isolates were found to be non-motile, testing negative for indole production, citrate use, catalase and oxidase. All the isolates fermented fructose, glucose, lactose and galactose confirming the heterofermentative or homofermentative nature of the genera for acid production [18].

Genus-specific PCR fingerprinting confirmed the 24 isolates to five *Bifidobacterium* strains (R2, R5, R6, S2 and S4) and two *Pediococcus* strains (N6 and P3), selected for acetic acid production studies. The 1500 bp 16S rDNA “amplicon” band was found to be a suitable molecular marker. The findings are in agreement Zolfaghari & Amin (2025) on the phylogenetic diversity of lactobacilli [19].

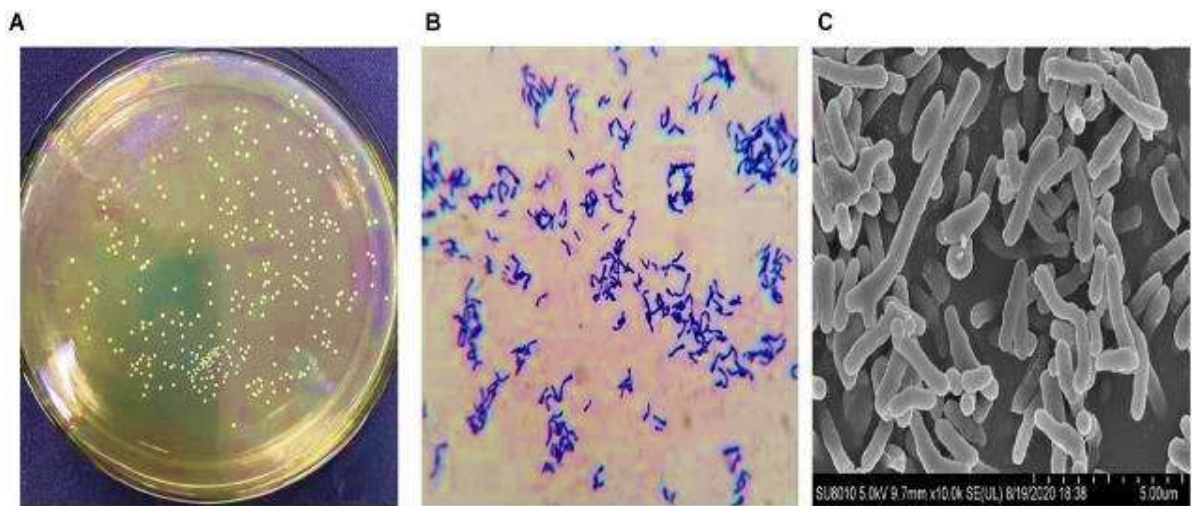


Fig 2. Phenotypic examination for *Bifidobacterium* on MRS Broth

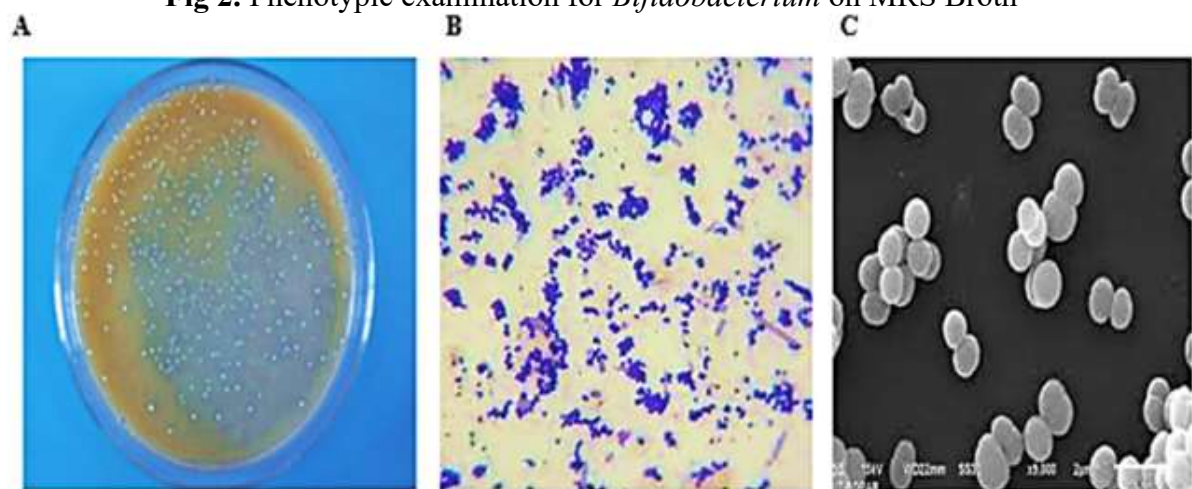


Fig 3. Phenotypic examination for *Pediococcus* on MRS Agar



Fig 4: PCR of *Bifidobacterium* primer which five isolates (1500 bp, agarose, DNA ladder, 16S/rDNA-1500-bp)

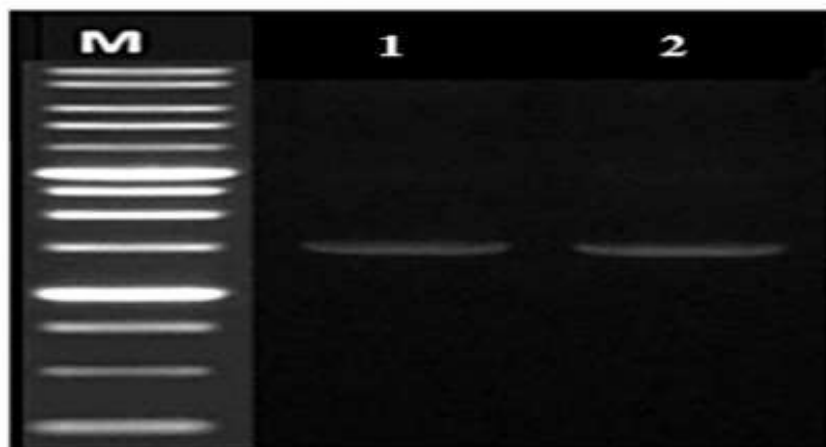


Fig 5: PCR of *Pediococcus* primer which five isolates (1500 bp, agarose, DNA ladder, 16S/rDNA-1500-bp)

3.2. Comparative Acetic Acid Production in Fruit Juice and Honey Media

These showed that fruit juice was better than honey for acetic acid fermentation by both bacteria (Fig 6). The *Bifidobacterium* isolate R2 produced 52.08 mg/L in fruit juice, which was remarkably high, compared with 34.99 mg/L (isolate R5) in the same medium. On the other hand, the *Pediococcus* isolate P4 produced the lowest yield; only 12.82 mg/L. This difference was attributed to the nature of fruit juice - with many sugars (glucose, fructose, and sucrose) that can be immediately utilized without the

involvement enzymatic invertase action for all of the oligosaccharides found in honey [20] The delectable micronutrients, nitrogenous substances, vitamins in the juice may have whetted the appetite of our fastidious friends the *Bifidobacteria*, engendering vast growth and α -accelerated activities that might have explained the high acetic acid titers observed in a similar fashion as for the *Pediococcus* in nutrient rich substrates [21]. That the yield by *Bifidobacterium* is significantly ($p < 0.05$) higher, suggests that this is a dog that the industrially hungry produce biligers would well have on their robustly fat hinds.

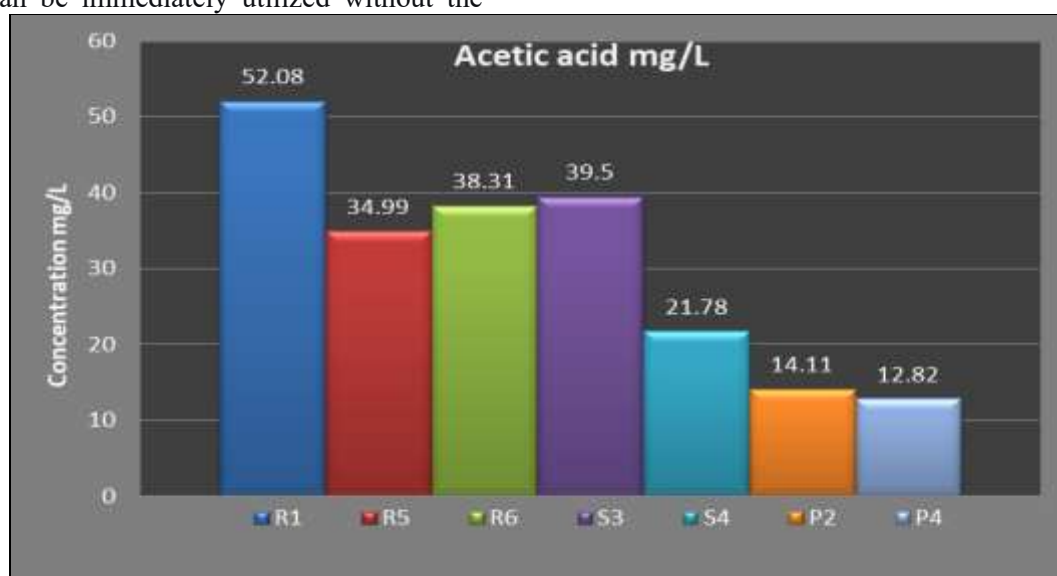


Fig 6. Schematic diagram of Acetic acid production by bacterial isolation nutrient media (mg/L)

3.3. Structural Verification and Purity Assessment

The successful biosynthesis and extraction of acetic acid was verified by full spectroscopic and

chromatographic characterisation. The FT-IR spectrum of the generated acid exhibited a broad O-H stretching band ($\sim 3000\text{ cm}^{-1}$), and a strong C=O stretching peak $\sim 1710\text{ cm}^{-1}$ which fitted that of analytical acetic acid (Fig 7), the characteristic feature of the carboxyl group [22]. HPLC peaks matched that of the standard perfectly, sample peak = standard, with calculated peak area consistent with titration approximates (Figure 8). Final

product yield of 87% pure produces not quite the analytical standard of 90%, but economically adequate product for food preservation, validating that ion-exchange chromatography would be an adequate method for purification of food grade bioproducts [23].

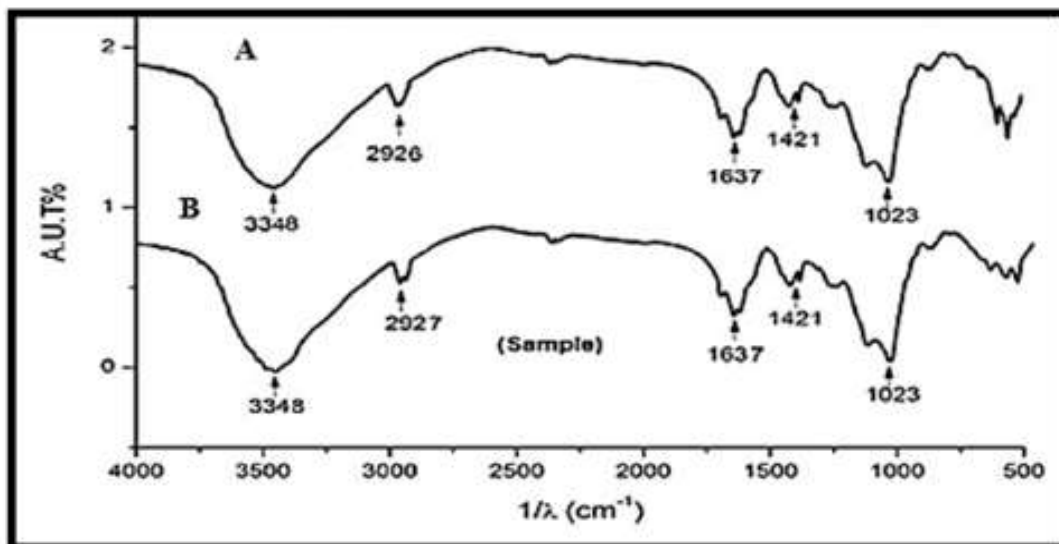


Fig 7. Diagnosis of Acetic acid standard (A) and in fruit juice (B) by FT-IR technology

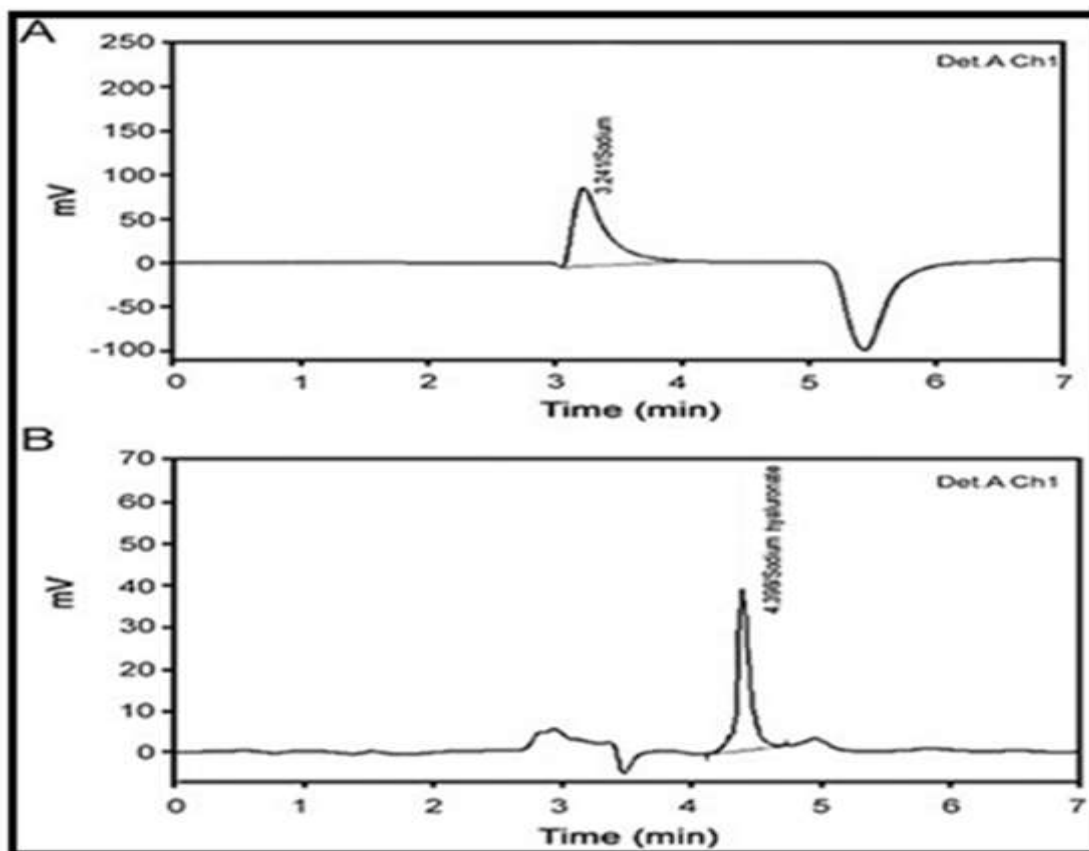


Fig 8. Identification of Acetic acid (A) and standard (B) by HPLC technique

3.4. Impact of Acetic Acid on Lipid Oxidation and Microbial Stability in Sheep Meat

The preservative action exerted by the acetic acid produced during fermentation in meat of sheep from de Bruyns et al. (2019) experiment was significant and concentration dependent. As shown by the chemical stability data (Table 3), protection against lipid oxidation was pronounced and significant ($p < 0.05$). By week 18 the control sample had a TBA value of 0.48 mg malonaldehyde/kg, which is approaching the sensory threshold for detection of rancidity.

Surprisingly, the final TBA value of a sample preserved with only 0.6% acetic acid was only 0.13 mg/kg. Similar data for peroxide values show that the 0.6% treatment stabilized the PV at a low level of 1.96 mEq/kg at week 18, compared with 2.53 mEq/kg in the control. This dual suppression of both endogenous primary (PV) and secondary (TBA) oxidation products suggests that acetic acid functions as a direct radical-scavenging antioxidant whilst also having the potential to concurrently act with endogenous meat constituents, a mechanism similar to that shown by Yilmaz et al. (2024) for microbe-derived metabolites [24].

Table 3. Chemical tests for P.V and TBA acid values of sheep meat

Tests	Sample														
	Control (0.0)%			Acetic acid (0.3 %)			Acetic acid (0.4 %)			Acetic acid (0.5 %)			Acetic acid (0.6 %)		
Time(week)	6	12	18	6	12	18	6	12	18	6	12	18	6	12	18
TBA mg/kg	0.16	0.27	0.48	0.13	0.16	0.19	0.12	0.14	0.16	0.10	0.13	0.15	0.09	0.11	0.13
P.V mEq/Kg	2.26	2.39	2.53	2.10	2.06	2.03	2.08	2.04	2.00	2.05	2.01	1.98	2.02	1.99	1.96

Elucidation of the microbiological findings in Table 4 demonstrates the antibacterial specificity in relation to acetic acid. Elimination of *C. perfringens* in all samples treated with 0.4% or greater acetic acid by week 18 is a significant safety advancement (see below). This spore-forming pathogen is notoriously tough to contend with, and their elimination is likely due to undissociated acetic acid molecules freely diffusing across the bacterial cell membrane and acidifying the cytoplasm, and interfering with vital metabolic functions

[25]. *Morganella* spp. inhibited enough so that it does not limit shelf-life, similar to the re-establishment of microbial communities by Bettini et al. (2025) [26]. The psychrotrophic *Flavobacterium* spp., a major cold spoilage agent, however, was more controlled than eradicated in the 0.6% samples, creeping up to 6520 cells/g in the control but holding at 2750 cells/g after treatment. This suggests that *Flavobacterium* spp. have no small measure of tolerance, but their growth is controlled enough as not to restrict the shelf-life [27,28]

Table 4. Microbial tests for *Clostridium perfringens* and *Flavobacterium* spp in sheep meat

Tests	Sample														
	Control (0.0)%			Acetic acid (0.3 %)			Acetic acid (0.4 %)			Acetic acid (0.5 %)			Acetic acid (0.6 %)		
Storage periods (week)	6	12	18	6	12	18	6	12	18	6	12	18	6	12	18
<i>Clostridium perfringens</i> × 10 ⁻⁶ (cell/gm)	1550	4900	2100	930	850	120	750	640	0	610	500	0	605	473	0

Flavobacterium
spp. $\times 10^{-8}$ (cell/gm)

1130 3200 6520 910 1200 1350 820 780 1490 650 710 1800 550 420 2750

3.5. Sensory Evaluation and Consumer Acceptability

The 0.6% acetic acid treatment received the highest scores from the expert panel, totalling a 'total acceptability' score of 93/100, the control managing only 79/100 as seen in Table 5 and Fig 9. The meat was also rated as superior for texture (19/20), which could possibly be attributed to a light protein alteration caused by surface adsorption of some of the acid that increases the meat's water holding capacity and tenderness. The high scores for taste and smell

(18/20) are news that the acid effectively inhibited the development of volatile rancid substances and odours without leaving any unpleasant vinegary residue, one of the major banes of acid-based preservation techniques. High values are crucial since validity is lent to the idea of preservation, proving it not only keeps the meat chemically intact, but also ensures a correspondingly beneficial value margin for the consumer during the actual eating process, in line studies involving natural additives for meat products [29, 30].

Table 5. Evaluation of meat samples (control and Acetic acid added)

Sample	Control No: 1	0.6% Acetic No: 2
Texture	17 b	19 a
Taste	16 b	18 a
Smile	15 b	18 a
Colour	15 c	19 c
Accept	16 b	19 a
Total	79 b	93 a

Note: Letter variations indicate significant differences between samples ($p > 0.05$) L.S.D = 0.258

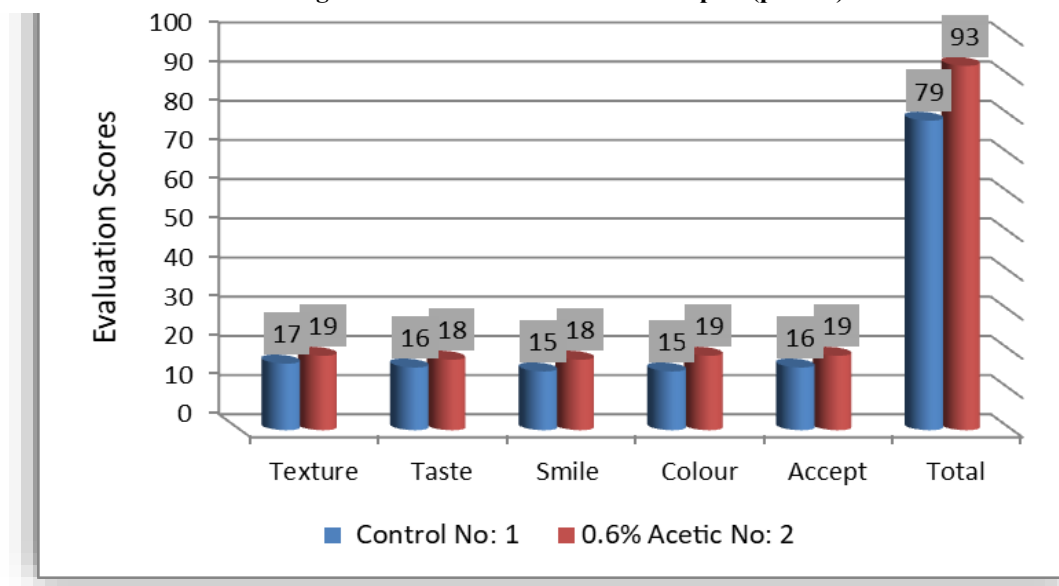


Fig 9. Sensory evaluation of the samples

4-Conclusions

We present here a complete from gadget-to-gut framework towards a circular bioeconomy approach to food preservation, covering

everything from the isolating of native probiotic bacteria to the application of that purified probiotic metabolite. Our results show that *Bifidobacterium* spp. are more prolific

Bacteriogenic food-grade acetic acid producers than any other probiotic reported, resulting when grown in the ubiquitous and inexpensive fruit juice medium. The application of this 0.6% bacteriogenic acetic acid onto sheep meat successfully produced a multi-hurdle preservation system with excellent chemical stability (low TBA and PV, signifying a good inhibition of lipid oxidation), stringent microbial safety (by inactivating the pathogen *C. perfringens* and effectively inhibiting cold-spoilage bacteria), and excellent sensory quality (93/100 acceptability rating over an 18 week shelf life). We advocate the scale-up of this probiotic-derived *biopreservative* technology as a safe, effective and palatable approach to help reduce food waste in general and preserve food from the unhealthy effects of synthetic preservatives in particular.

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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استفاده از باکتری‌های مفید برای افزایش ارزش غذایی غذاهای غنی از پروتئین با تولید ترکیبات

آلی سالم

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عسل.

در این مطالعه، گونه‌های پدیوکوکوس و بیفیدوباکتریوم ایزوله شده در آزمایشگاه، اسید استیک را از عسل و آب میوه غنی از مواد مغذی تولید کردند. بیفیدوباکتریوم عملکرد بهتری نسبت به پدیوکوکوس (۵۲.۰۸ میلی گرم در لیتر) داشت. گوشت گوسفند غنی شده با اسید استیک پس از ۱۸ هفته، معیارهای بهبود یافته‌ای را نشان داد (TBA: 0.13 میلی گرم در کیلوگرم، P.V: 1.96 میلی اکی والان در کیلوگرم). کلستریدیوم پرفرنژنس وجود نداشت، در حالی که گونه‌های فلاوباکتریوم شناسایی شدند (۲۷۵۰ سلول در گرم). ارزیابی حسی متخصصان، امتیاز ۹۳ از ۱۰۰ را نشان داد که نشان دهنده مقبولیت بالا است. افزودن اسید استیک، ماندگاری در یخچال را افزایش داد و به مسائل مربوط به نگهداری در دمای غیر استاندارد پرداخت و کیفیت گوشت و مشخصات تغذیه‌ای آن را بهبود بخشید. بر اساس این اصل، هدف این مطالعه دستیابی به اهدافی با ارزش علمی و عملی و تأثیر مثبت بر سلامت محصول و در نتیجه سلامت انسان بود. بر اساس این اصل، هدف این مطالعه استفاده از یکی از اسیدهای آلی سالم است که نقش برجسته و مؤثری در حفظ مواد غذایی مانند گوشت گوسفند و نگهداری آن برای مدت طولانی در دماهای مختلف و همچنین بهبود کیفیت و ویژگی‌های حسی آن مانند طعم، رنگ، بافت و بو دارد.

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