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- processing of meat raw materials;
- improvement of technologies for meat product manufacture;
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TRENDS IN THE HORMONE AND ANTIBIOTIC USE FOR CATTLE FATTENING IN BANGLADESH

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Keywords: *beef cattle, beef consumption, impact of residue, public health, withdrawal period*

Abstract
The study was conducted to identify trends in the use of hormones and antibiotics, and traditional practices in cattle fattening in the southwest coastal region of Bangladesh. Data were collected from 150 farmers involved in beef cattle fattening through a survey questionnaire. The average numbers of cattle and beef cattle per household were 4.27 ± 1.94 and 3.54 ± 1.31, respectively. An average of 44.67 percent of the beef farmers selected indigenous (zebu) cattle and the rest of the farmers kept a variety of crossbred cattle for fattening. The average age of cattle at the start of fattening was 21.61 ± 8.07 months, the average duration of fattening was 9.71 ± 4.29 months, and farmers marketed cattle at an average weight of 285.50 ± 50.80 kg. The highest percentage (34.67%) of farmers ate beef once a week and 5.33 percent of farmers never ate beef. Among beef farmers, 53.33% and 62.67% applied hormones and antibiotics, respectively, to their beef cattle. The highest percentage of farmers used hormones (33.87%) and antibiotics (29.79%) once a week and the rest of the farmers used them at various intervals. The majority of farmers did not follow any withdrawal period before slaughtering beef cattle for application of hormones (85.48%) and antibiotics (83.58%), whereas the remaining farmers maintained different withdrawal periods. It can be concluded that more than half of the farmers applied hormones and about two-thirds of the farmers used antibiotics for beef fattening, and most of the farmers did not follow the recommended withdrawal periods before slaughter, which is of public health concern.

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Introduction

In Bangladesh, the fattening of cattle holds great potential for creating jobs and revenue for the impoverished rural population, particularly tiny, marginal, and landless farmers. It is an alternate strategy for eradicating rural poverty. Small farmers in Bangladesh are increasingly turning to cattle fattening as a means of producing high-quality beef [1]. Additionally, it is a tool for improving rural impoverished people's livelihoods and generating revenue [2]. It was discovered that 8.71 million metric tons of meat were generated in Bangladesh in the fiscal year of 2022–2023 as a result of recent efforts to fatten cattle and raise broilers [3]. The farmers purchase cattle three to six months before the Muslim festival of Eid-ul-Azha, after which they fatten and sell them. Bangladeshi female farmers have been actively involved in and have supported beef fattening initiatives in the nation's rural areas in recent years. The local banks, NGOs, and other credit groups provide loans to female farmers. The cattle fattening industry provided between 30 and 60 percent of the income for rural farmers [4].

Feed additives and growth boosters are supplied into Bangladesh by pharmaceutical companies and foreign marketing agencies, luring farmers to utilize them for animal fattening. According to Islam et al. [4], the majority of cattle brought for sale as sacrifice animals during *Eid-ul-Azha* are purportedly fattened by dishonest cattle traders, who disregard the livestock department's scientific formula, for quick profits and rapid live weight growth. The Fish Feed and Animal Feed Act 2010 of Bangladesh [5] bans the use of steroid hormones and antibiotics, which are forbidden both domestically and internationally, for animals in Bangladesh. A type of steroids is used to quickly increase the weight of the sacrificed animals in the days leading up to *Eid*, as they aim to increase revenue from the cattle trade during the celebration.

It is estimated that 50% of antibiotics used worldwide are used to stimulate animal growth [6]. When administering antibiotics to animals raised for food, care must be taken to ensure that the consequences for humans who eat foods of animal origin are also taken into account [7]. The overuse of these antibiotics in animal husbandry has re-

sulted in the buildup of them in animal tissues in the muscles, heart, liver, and kidney beyond the relative maximum residue levels (MRLs) [8]. The main reasons why drug residues build up in animals raised for food are overdosing, not continuing treatment, inadequate monitoring of withdrawal times, and the use of illegal antibiotics for commercial animal care [9]. One of the hazards to the health of both human and animal bodies is the antibiotic residue. Antibiotic resistance in pathogens affects both humans and animals when farmers and veterinarians improperly administer antibiotics without adhering to the withdrawal period [10]. These issues include teratogenicity, immunopathological effects, carcinogenicity, estrogenic effects, neurotoxicological effects, toxicity, transfer of antibiotic-resistant bacteria to humans [11]. The following objectives were taken into consideration when designing this study, which focused on beef fattening in Bangladesh's southwest coastal districts.

Objectives of the study:

1. To find the trends of using hormones and antibiotics in beef cattle fattening in southwest regions of Bangladesh.
2. To identify the traditional beef cattle fattening program in the southwest coastal region of Bangladesh.

Objects and methods

Design of the study

Data for the current study was collected from the respondents (beef cattle farmers) through door-to-door interviews. Its purpose was to investigate the patterns of hormone and antibiotic use in the Bangladesh's southwest coastal region.

Locations and sampling of the beef cattle farmers

Three areas in the Bangladesh's southwest coastal region were used for the study. These were the randomly chosen region of Tala from Satkhira, Fakirhat from Bagerhat, and Dumuria from Khulna districts. A total of 150 farmers who were involved in fattening of cattle and were either ready or willing to provide information were questioned. From each site, 50 farmers were chosen at random. Sampling of the beef cattle farmers to collect the information is shown in Table 1.

Table 1. Sampling of the beef cattle farmers

Sl. No.	Name of locations	No. of cattle farmers
1	Dumuria, Khulna	50
2	Tala, Satkhira	50
3	Fakirhat, Bagerhat	50
Total		150

Preparation of the interview schedule (questionnaire)

A meticulously crafted interview schedule was devised to elicit pertinent and valuable information from the beef cattle farmers. It had both closed-ended and open-ended questions, all of which had the straightforward format. The questions were designed to be quite simple so that the respondents could answer them with accuracy and ease of understanding. The interview schedule (questionnaire) was pretested with a few farmers of beef cattle once it was prepared. Pre-testing entailed a set of exercises intended to assess a survey instrument's ability to collect the required data; the interview schedule was then multiplied in its ultimate form to facilitate data collection.

Collection and analysis of data

Face-to-face interviews with respondents were conducted in accordance with the interview schedule in order to gather data for this study. The goals of the study were described to the respondents before asking any questions, and we asked for their cooperation so that they could provide us with truthful and accurate information. The beef cattle farmers responded based just on their memories because they lacked a written document. Following each interview, the data sheets were examined and confirmed to ensure that the respondents' responses had been accurately recorded. Following the end of the interview, a respondent received appropriate gratitude. To ensure ease and accuracy in achieving the goals, a basic statistical method was employed for data analysis. The data was analyzed using IBM SPSS statistics.

Results and discussion

Number of cattle per household

According to the data in Table 2, there were 3.56 ± 1.58 cattle (including beef, dairy and others) on average per household at the Dumuria area, compared to 3.08 ± 1.16 beef cattle. In the Tala region, there were an average of 4.18 ± 1.90 cattle (including beef, dairy and others), with an average of 3.60 ± 1.36 beef cattle per household. In the Fakirhat region, there were 5.06 ± 2.03 cattle (including beef, dairy and others) and 3.94 ± 1.30 beef cattle per household, respectively.

As per an alternative survey, 42% of farmers raised between two and five beef cattle, 33% between six and nine beef cattle, 18% between ten and twelve cattle, and just 7% raised over 12 cattle for fattening [1]. According to Begum et al. [12], just 3% of farmers raised more than eight cattle, while 70% of farmers raised one to four cattle, 27% raised five to eight cattle. Similarly, 79% of farmers grew 2–5 cattle, 17% raised 6–9 cattle, and only 3% raised 12 or more cattle, as reported by Islam et al. [4].

Table 2. Average number of cattle per household in three different study areas

Locations of the study	Mean ± SD	
	Total cattle (including beef, dairy and others)/ household	Beef cattle/ household
Dumuria of Khulna district	3.56 ± 1.58	3.08 ± 1.16
Tala of Satkhira district	4.18 ± 1.90	3.60 ± 1.36
Fakirhat of Bagerhat district	5.06 ± 2.03	3.94 ± 1.30
Total	4.27 ± 1.94	3.54 ± 1.31

Genotypes of fattening cattle

Data in Table 3 indicate that the highest percentage (44.67%) of the beef cattle that farmers reared were indigenous (zebu) cattle followed by indigenous × Holstein Friesian crossbred (29.33%), indigenous × Shahiwal crossbred (18.67%), indigenous × Red Sindhi (5.33%) and indigenous × Jersey crossbred (2.0%) cattle.

Table 3. Genotypes of fattening cattle reared by the beef cattle farmers

Genotypes of cattle	Frequency	Percent
Indigenous (zebu)	67	44.67
Crossbred (indigenous × Holstein Friesian)	44	29.33
Crossbred (indigenous × Shahiwal)	28	18.67
Crossbred (indigenous × Red Sindhi)	8	5.33
Crossbred (indigenous × Jersey)	3	2.00
Total	150	100.00

As per Hasan et al. [13], indigenous cattle were raised by 44.44% of farmers and crossbred cattle by 24.44%, while 31.11% of farmers raised a combination of both, which is consistent with the current results. Islam et al. [4] reported that 42.7% of farmers selected indigenous cattle, while 57.3% selected crossbred with the intention of fattening the animals, which is also in agreement with the result of the present study. According to Kamal et al. [14], 26.3% of the farmers chose indigenous cattle, 32.5% chose crossbreeds, and 41.3% chose both local and crossbred cattle for their beef cattle farming. According to Rahman et al. [15], around 60% of farmers fattened their cattle by using both indigenous (zebu) and crossbred animals; 28% used indigenous cattle and 12% used crossbreds. According to Hossain et al. [16], 88% of cattle were crossbred and only 12% were indigenous cattle. The present finding does not match the findings of other researchers [14,15,16]. These differences may be due to different study locations, consumers' demand of the locations, and socioeconomic status of beef cattle farmers.

Age of cattle at the beginning of fattening

The data in Table 4 shows that maximum percentage (34.7%) of cattle farmers started the fattening program with cattle between 12 and 18 months of age. Among the remaining farmers, 24.0% started with cattle between 19 and 24 months of age, 20% with cattle between 25–30 months of age, 8.0% with cattle below 12 months of age and between 31 and 36 months of age. Only 5.3% of the farmers started fattening cattle above 36 months of age.

Table 4. Age category of the cattle for fattening purpose

Age of cattle at the beginning	Frequency	Percent	Mean ± SD
<12 months	12	8.0	
12–18 months	52	34.7	
19–24 months	36	24.0	
25–30 months	30	20.0	21.61 ± 8.07
31–36 months	12	8.0	
>36 months	8	5.3	
Total	150	100.0	

Composition of feeds for beef cattle

Different components of feeds for beef cattle are presented in Table 5. It is shown in Table 5 that the beef cattle were fed composite feeds including rice straw, green grasses, concentrate mix and urea molasses treated straw (UMS) in different combinations. Green grasses comprised of natural grass (*Cynodon dactylon*), Napier (*Pennisetum purpureum*) and German (*Echinochloa polystachya*) grasses. Concentrate mix comprised of maize (50%), wheat bran (22%), soybean meal (25%), dicalcium phosphate (2%) and common salt (1%), and urea molasses treated straw (UMS) comprised of urea (3%), molasses (14%) and rice straw (83%). The results revealed that more than half (50.67%) of the beef cattle farmers fed their beef cattle with a combination of green grasses, rice straw and concentrate mix followed by a combination of green grasses and concentrate mix (17.33%), green grasses and rice straw (15.33%), green grasses, UMS and concentrate mix (9.33%), and rice straw and concentrate mix (7.33%).

According to Mamun et al. [17], the majority of beef cattle farmers (58.3%) provided both cultivated fodder and compound feed. The remaining 1.7% of farmers fed roadside grass to their cattle, while 5% fed both cultivated fodder and cultivated grass, 10% fed both cultivated fodder and mixed feed, and 23.3% fed both cultivated fodder and compound feed. In contrast to straw (17.78%) and planted fodders (26.67%), the majority of farmers provided roadside grass (55.56%) as the source of forages [13]. According to Hossain et al. [16], the majority of farmers (83%) fed their cattle with cultivated fodder, whereas only 17 percent fed their cattle with both roadside grass and cultivated fodder. A number of researchers have recently suggested that feeding cattle on grass-based rations could be a viable choice for fattening cattle [18,19]. For the purpose of fattening cattle, the remaining farmers (44.44%) provided both ready mix and homemade feed mixture. Rashid et al. [20] stated that 55% of feeds were roughage and 45% were concentrate. In their study, 70% of farmers were dependent on natural feed and 30% were solely dependent on market feed. According to Kamal et al. [14], 3.8% of farmers provided only concentrate, whereas 96.3% of farmers provided both roughage and concentrate. In contrast to Buza and Holden [21], who reported that 97.6% of Pennsylvanian survey respondents fed a total mixed ration (TMR), the farmers surveyed by Kamal et al. did not employ any TMR.

Table 5. Composition of feeds for beef cattle

Feed components	Frequency	Percent
Green grasses* + Rice straw	23	15.33
Green grasses + Rice straw + Concentrate mix**	76	50.67
Rice straw + Concentrate mix	11	7.33
Green grasses + Concentrate mix	26	17.33
Green grasses + UMS*** + Concentrate mix	14	9.33
Total	150	100.00

* Green grasses comprised of natural grass (Cynodon dactylon), Napier (Pennisetum purpureum) and German (Echinochloa polystachya) grasses.
** Concentrate mix comprised of maize (50%), wheat bran (22%), soybean meal (25%), dicalcium phosphate (2%) and common salt (1%).
*** UMS (urea molasses straw) comprised of urea (3%), molasses (14%) and rice straw (83%).

Duration of cattle fattening

Data in Table 6 indicate that average duration for cattle fattening was 9.71 ± 4.29 months. The highest percentage of the farmers (32.0%) raised beef cattle for a period of 9 to 12 months, followed by 6 to 8 months (30.0%), above 12 months (22.0%) and 3 to 5 months (16.0%). In a similar vein, Kamal et al. [14] discovered that 16.3% of farmers raised fattening animals for three months or less, while the highest percentage of farmers (35%) raised cattle for three to six months, 31.3% for six to twelve months, and the remaining farmers raised cattle for more than twelve months. According to Ahmed et al. [22], 79.1% of respondents thought that the best duration to fatten cattle was between three and six months prior to Eid-ul-Azha, 4.7% thought that the period should be between six and twelve months, and 16.3% said that the period should be more than twelve months. Since most people in Bangladesh are Muslims, Hasan et al. [13] claim that Eid ul Azha is a significant Islamic festival in this country. On the day of Eid ul Azha, devout Muslims offer sacrifices of cattle, buffalo, goats, or sheep. In order to provide their cattle on the cattle market before Eid ul Azha, the majority of farmers (57.78%) fattened their livestock before the festival. Throughout the year, just 42.22% of farms were involved in fattening operations.

Table 6. Duration of cattle fattening

Duration of cattle fattening	Frequency	Percent	Mean ± SD
3–5 months	24	16.0	
6–8 months	45	30.0	
9–12 months	48	32.0	9.71 ± 4.29
>12 months	33	22.0	
Total	150	100.0	

According to Islam et al. [4], the majority of farmers (53.3%) begin fattening cattle before Eid-ul Azha, with the remaining farmers (47%) fattening cattle all year long. According to the fattening period of the study, 44% of the farmers who fattened beef cattle did so for three to six months, 7% did it only before Eid-ul-Azha, and 24% did so all year long [1]. According to Begum et al. [12], 60% of cattle farmers fattened their animals within three months of Eid ul Azha. Rahman et al. [15] stated that 44.7% of beef producers fattened their cattle for three months, while the remaining farmers fattened their cattle for six months or a year. The majority of livestock caretakers (35%) periodically fatten their cattle for additional cash in a short amount of time [20]. Similar findings were published by Foeken et al. [23], who discovered that urban farmers constantly made an effort to improve their financial situation. Around 65% of farmers carry out the fattening all year long. Most respondents (44.7%) fattened cattle for three months, whereas the remaining respondents fattened cattle for six months or a year, according to Rahman et al. [15].

Marketing weights of fattened cattle

Different weight classes of beef cattle at marketing are shown in Table 7. Data in Table 7 indicated that the highest percentage (30.0%) of farmers marketed beef cattle at a weight of between 201 and 250 kg and the lowest percentage at a weight of above 400 kg. The average marketing weight of cattle was 285.50 ± 80.30 kg.

Table 7. Marketing weights of fattened cattle

Marketing weight (kg)	Frequency	Percent	Mean ± SD
150–200	24	16.0	
201–250	45	30.0	
251–300	24	16.0	
301–350	21	14.0	285.50 ± 80.30
351–400	21	14.0	
>400	15	10.0	
Total	150	100.0	

Consumption frequencies of beef

The frequency of beef consumption by farmers is shown in Table 8. The data presented in Table 8 revealed that the highest percentage (34.67%) of beef cattle farmers consume beef once a week and the lowest percentage (3.33%) consume it daily. A study conducted by Jahan et al. (2008) indicated that the overall average monthly consumption of beef was about 1.86 kg per household in Bangladesh.

Table 8. Consumption frequencies of beef

Frequencies	Number of farmers	Percent
Once daily	5	3.33
2 to 5 times daily	19	12.67
Once a week	52	34.67
Once every two weeks	37	24.67
Once a month	18	12.00
Once a year	11	7.33
No consumption	8	5.33
Total	150	100.00

Use of hormones and antibiotics

Data collected from three different locations in the southwest coastal region of Bangladesh indicated that more than half (53.33%) of the beef farmers used hormones and 62.67% of the farmers applied antibiotics for fattening cattle (Table 9). According to Islam et al. [4], 70.6% of respondents utilized anabolic steroids in place of growth hormones, while the remaining respondents did not use any form of growth hormones, which is consistent with the present findings. Islam et al. [4] discovered that 95.3% of the farmers used feed additives (antibiotics) to fatten cattle, while the remaining 4.7% used no feed additives at all and the percentage was higher than the current result. On the other hand, 95% of the farmers who fattened their cattle did not utilize any growth promoters during the fattening process, and just 5% of them

used steroids as a growth promoter [13], which is different from the present result. Only 22.22% of the farmers surveyed utilized antibiotics and growth promoters; the remaining farmers did not use any growth promoters throughout the fattening phase [13]. According to Rahman et al. [15], around 34.7% of farmers in the rural areas used beef fattening hormone tablets.

Table 9. Percentage of the farmers used hormones and antibiotics in cattle fattening

Categories	Hormones		Antibiotics	
	Frequency	Percent	Frequency	Percent
Used	77	51.33	94	62.67
Not used	73	48.67	56	37.33
Total	150	100.00	150	100.00

According to Barman et al. [12], just 7% of farmers employed growth hormones to make their animals fatter in order to produce meat. Low-income farmers were also reported by Islam et al. [4] to use anabolic steroids more frequently. As per Kamal et al. [14], 58.8% of the farmers used steroids as a growth promoter, while the remaining farmers did not use any form of growth promoters during the fattening phase. According to Rahman et al. [15], 34.7% of farmers utilized hormone pills that fatten cattle.

Advisers on the use of hormones and antibiotics

Cattle farmers receive advice on the use of hormones and antibiotics to fatten cattle from various sources. The data presented in Table 10 shows that maximum 59.68% and 45.74% of the farmers were advised to use hormones and antibiotics, respectively, by animal health workers. The second highest percentage of cattle farmers were advised by local doctors to use hormones (27.42%) and antibiotics (25.53%). According to Islam et al. [4], around 49% of respondents used vitamin mineral premix, 26% enzyme, 12% antibiotics, and 13% anabolic steroids for fattening cattle. They also claimed that among advisors of using feed additives in beef fattening 25% were farmers themselves, 50% nearby farmers, 17% NGO employees, and 8% veterinary representatives. According to Kamal et al. [14], it was discovered that 28.8% of farmers were advised to use steroids by beef businessmen, 15% by feed dealers, 8.85% by neighbors, 2.5% by NGO workers, and 3.8% by veterinary medical representatives.

Table 10. Advisers on the use of hormones and antibiotics for beef cattle fattening

Categories	Hormones		Antibiotics	
	Frequency	Percent	Frequency	Percent
Local doctors	17	27.42	24	25.53
Veterinary doctors	0	0.00	15	15.96
Animal health workers	37	59.68	43	45.74
Farmers themselves	8	12.90	12	12.77
Total	62	100.00	94	100.00

Farmers' perceptions of the impact of hormone and antibiotic use in beef cattle on human health

Information on farmers' perceptions of the impact of the hormone use in beef cattle on human health is presented in Table 11. Data in Table 11 shows that the highest percentage (45.33%) of beef cattle farmers did not know about the effects of hormones used on human health, while 14.0% of farmers said that there were no effects, 11.33% said hormones could cause toxicity, 8.67% said they could cause obesity, 7.33% mentioned early maturity and failure of therapy and 6.0% said they could cause breast cancer in humans. Hasan et al. [13] stated that just 22.22% of farmers were aware of the potential health risks associated with steroids, while the remaining 77.78% were unaware of these risks. According to Barman et al. [24], 93% of rural farmers want to increase their profits quickly, despite the fact that 50% of them are unaware of the importance of managing livestock health. According to Rashid et al. [20], 99.5% of people do not believe that using hormones has negative effects. Kamal et al. [14] stated that 98% of farmers believed that steroids had a favorable effect on productivity or growth rate of beef cattle. Merely 30% of farmers were aware of the potential health risks associated with steroids [14].

Table 11. Farmers' perceptions of the impact of the hormone use in beef cattle on human health

Types of impact	Frequency	Percent
No impact	21	14.00
Failure in therapy	11	7.33
Toxicity	17	11.33
Obesity	13	8.67
Early maturity	11	7.33
Breast cancer	9	6.00
Don't know	68	45.33
Total	150	100.00

Data for farmers' perceptions of the impact of the antibiotic use in beef cattle on human health is shown in Table 12. The results revealed that the highest percentage of the farmers (54%) said that they did not know about the impact of antibiotics in beef cattle raising on human health. On the other hand, 14.67% of farmers stated that there is no impact, 12% mentioned failure in therapy, 10.0% said that they are a cause of toxicity and 9.33% farmers stated that they can create multi-drug resistance in human.

Table 12. Farmers' perceptions of the impact of the antibiotic use in beef cattle on human health

Types of impact	Frequency	Percent
No impact	22	14.67
Failure in therapy	18	12.00
Multi-drug resistance	14	9.33
Toxicity	15	10.00
Don't know	81	54.00
Total	150	100.00

Purposes of the hormone and antibiotic usage in beef cattle by the farmers

Purposes of the hormone usage in beef cattle by the farmers are presented in Table 13. More than half of the farmers (58.06%) used hormones as a growth promoter followed by 35.48% who used them for quick fattening and the rest 6.45% used for treatment of sick animals.

Purposes of the antibiotic usage in beef cattle by the farmers are presented in Table 14. The highest percentage (36.17%) of farmers applied antibiotics for the treatment of sick animals. The rest 31.91% used antibiotics for growth promotion, 24.47% as disease preventive measures and 7.45% of farmers used them for improving feed efficiency.

Table 13. Purposes of the hormone usage in beef cattle by the farmers

Purposes	Frequency	Percent
Treatment of sick animals	4	6.45
Growth promotion	36	58.06
Quick fattening	22	35.48
Total	62	100.00

Nichols et al. [25] stated that the use of steroid implants in an intensive beef cattle production system boosted average daily gain by 15 to 25% and feed efficiency by 10 to 15%; however, longer-term usage of steroid implants was associated with a decrease in marbling. Growth implants increased ($P < 0.05$) average daily gain by 11.8 to 20.5% in steers, according to Platter et al. [26]. According to Haque and Sarker [27], Bangladesh used a wide variety of steroids extensively for cattle and poultry. Asem-Hiablie et al. [28] found that growth implants were utilized for the production of beef cattle on an average of 30% of US ranches in the northwest and southwest.

Table 14. Purposes of the antibiotic usage in beef cattle by the farmers

Purposes of using antibiotics	Frequency	Percent
Treatment of sick animals	34	36.17
Disease preventive measures	23	24.47
Growth promotion	30	31.91
Improvement of feed efficiency	7	7.45
Total	94	100.00

Sources of hormones and antibiotics for beef cattle

Sources of hormones and antibiotics for applying in beef cattle are presented in Table 15. Data revealed that the agents of pharmaceutical companies were the highest (41.94%) source of hormones; however, for antibiotics, the highest source (44.68%) was local veterinary pharmacy. The second highest sources for hormones and antibiotics were local veterinary pharmacy and animal health workers (29.03% and 28.72%, respectively), followed by animal health workers and agents of pharmaceutical companies (22.58% and 20.21%, respectively), and beef cattle buyers (6.45 and 6.38%, respectively).

Table 15. Sources of hormones and antibiotics for beef cattle

Sources	Hormones		Antibiotics	
	Frequency	Percent	Frequency	Percent
Local veterinary pharmacy	18	29.03	42	44.68
Animal health workers	14	22.58	27	28.72
Agents of pharmaceutical companies	26	41.94	19	20.21
Beef cattle buyers	4	6.45	6	6.38
Total	62	100.00	94	100.00

According to Islam et al. [4], around 49% of respondents used vitamin mineral premix, 26% enzyme, 12% antibiotics, and 13% anabolic steroids for fattening cattle. They [4] also claimed that advisors for using feed additives were farmers themselves (25%), farmers from nearby farms (50%), NGO employees (17%), and veterinary representatives (8%).

Pattern of applying hormones and antibiotics in beef cattle

Data on pattern of applying hormones and antibiotics in beef cattle is shown in Table 16. The highest percentage of beef cattle farmers used hormones and antibiotics once a week (33.87% and 29.79% for hormones and antibiotics, respectively). For hormones, 19.35% of farmers used them once in two weeks followed by farmers who used them once a month (17.74%) and once a year (9.68%). In case of antibiotics, 25.53% of farmers used them daily followed by farmers who used them once a month (12.77%), once in two weeks (11.70%), 2 to 3 times a week (10.64%), once in six months (7.45%) and once a year (2.13%).

Table 16. Pattern of applying hormones and antibiotics in beef cattle

	Hormones		Antibiotics	
Pattern of use	Frequency	Percent	Frequency	Percent
Daily	2	3.23	24	25.53
2–3 times a week	5	8.06	10	10.64
Once a week	21	33.87	28	29.79
Once in two weeks	12	19.35	11	11.70
Once a month	11	17.74	12	12.77
Once in six months	5	8.06	7	7.45
Once a year	6	9.68	2	2.13
Total	62	100.00	94	100.00

Withdrawal period of hormone and antibiotic application at cattle slaughter

Data for the withdrawal period of hormones and antibiotics application at beef cattle slaughter is shown in Table 17. Data revealed that the majority of the farmers did not follow any withdrawal period both for hormones (85.48%) and antibiotics (83.58%) at slaughter. The second largest percentage of farmers (6.45%) maintained the 7-day withdrawal period for hormones followed by the 15-day

(4.84%) and 3-day (3.23%) withdrawal periods. In case of antibiotics, the second longest withdrawal period (7.46%) was 7 days followed by 3 days (5.97%) and 15 days (2.99%). Alarmingly, according to Kamal et al. [14], 55.3% of farmers had stopped using steroids right before marketing, 27.7% had stopped using them before slaughtering, and 17% had stopped using them before a month of marketing.

Table 17. Withdrawal period of hormone and antibiotic application at beef cattle slaughter

Withdrawal period (days)	Hormones		Antibiotics	
	Frequency	Percent	Frequency	Percent
No withdrawal time	53	85.48	56	83.58
3	2	3.23	4	5.97
7	4	6.45	5	7.46
15	3	4.84	2	2.99
30	0	0.00	0	0.00
Total	62	100.00	67	100.00

Conclusion

The results revealed that small and marginal farmers are generally rearing beef cattle in the southwest coastal region of Bangladesh and most of them raise indigenous (zebu) cattle for fattening. The average age of beef cattle at the beginning was 21.61 months, animals were marketed at an average live weight of 285.50 kg and reared for an average of 9.71 months. Farmers fed their beef cattle with different combinations of paddy straw, green grasses, concentrate mix and urea-molasses treated straw. More than half and nearly two-thirds of farmers used hormones and antibiotics, respectively, in beef cattle at varying frequencies. Almost half of the farmers did not know the negative effects of the hormone and antibiotic use in beef cattle on human health, which indicates that there is a need to create awareness among cattle farmers about human health. Most farmers did not follow the withdrawal periods for hormones and antibiotics at slaughter, which is a public health concern. It can be concluded that in the southwest coastal region of Bangladesh, small- and medium-scale cattle farmers apply hormones and antibiotics at different frequencies without following a withdrawal period. This problem can be solved by supporting cattle farmers by creating awareness and providing an appropriate technology for cattle fattening.

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HACCP OPERATION IN TWO LATIN AMERICAN MEAT PACKERS: CASE STUDY

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Keywords: *meat exporters, food safety, Latin American packers, HACCP*

Abstract
The hazard analysis and critical control point (HACCP) system is a protocol aimed to guarantee food safety, especially in companies dedicated to meat processing. Companies in food international trade are subjected to intense inspection and verification processes, and international standard certifications have become a key factor in global trade. The purpose of this study was a comparative analysis of HACCP certification in the Mexican and Chilean meat packers that send meat to international markets by a field inspection of each to identify opportunities for improvement. International trade standards along with packers' expectations and needs to be fulfilled to attain a place in world markets were bases for this study. Guidelines for field inspection came from the HACCP protocol. Field data were derived from 15 indicators — five intrinsic to the HACCP plan and ten external. The Chilean packer was superior to the Mexican packer in terms of many indicators. Thus, the first one had a chance for a better position in international markets than the latter. It was concluded that the HACCP audit is an excellent tool to measure the suitability of meat packers in achieving a place as a supplier and remaining in the international food trade.

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Introduction

Food safety is a constant concern worldwide. Human health institutions and governments are constantly looking for the most appropriate forms of supervision throughout the entire food production chain. It presents the provision of varying quality management tools that emphasize product standardization [1]. The HACCP system has a worldwide recognition as a preventive system for food safety. It is based on measuring hazards, estimating risks, and establishing specific control measures aimed to prevent and control. Shuvo et al. [2] indicated that its implementation by the food industry enables it not only to produce safe food but also to demonstrate how food safety issues are designed and applied objectively and transparently. HACCP is a tool with a systematic approach that is based on the application of specific science to each of the reported hazards to achieve food safety.

According to Borodin et al. [3], for a food business to be competitive and remain in the market, it is necessary to monitor hazards and control critical parameters determined at each stage of production. In the case of companies dedicated to meat products, this monitoring must also be carried out from raw materials and auxiliary materials up to the final product. De Oliveira et al. [1] stated that some prerequisites, such as Good Manufacturing Practices (GMPs) and Sanitation Standard Operating Procedures (SSOPs), should be established before HACCP implementation and operation. Their function is to keep hazards in the facility under control, whereas HACCP focuses on managing specific hazards within processes. In the meat industry, the application of GMPs and SSOPs impacts the production of safe and high-quality products. Therefore, the strict application of these regulatory guidelines at all stages guarantees the production of meat products that meet the quality levels required by consumers [4]. Another factor of interest refers to the hygiene and habits of employees to avoid contamination of products, which can only be achieved through Good Hygiene Practices (GHPs) and training as a fundamental part of an integrated program [5]. ADAFSA [6] mentioned that promoting GMPs and achieving them require auditing processes throughout the supply chain. Audits report risks that are still present whether in facilities, equipment, or work areas.

The meat industry has undergone substantial changes in recent years due to the development of new technologies in various areas, such as primary production, slaughtering, dressing, and meat processing [7]. The OECD/FAO [8] indicated that production is expected to increase 16% by 2025 because of increased meat demand in developing countries due to economic growth, increased consumer purchasing power, and increased knowledge of food composition and properties. Consequently, production is focused on the main challenges in meat safety that are related to traditional hazards, as well as new or emerging ones. It is equally important to identify food safety objectives based on risk assessment in the production and processing of meat and systematic food management as proposed by the HACCP system [9].

As a result, inspection has a relevant role in the control of safety. In European countries, modernization is being applied both for the improvement of livestock health and processing. The notable change is that nowadays a risk-based inspection is used, which is in line with a safety assurance system. Factors such as existing trade agreements with third countries, costs involved in the inspection process, inadequate food chain information, and the reluctance of inspectors have formed a wall of obstacles to be faced. Improvement of the components is necessary for the modernization of inspection systems to be achieved, thereby reducing the workload [10]. Laukkanen-Ninios et al. [11] pointed out the importance of continuous meat inspection based on scientific and practical reasons, taking into account the scientific point of view and risk management. For this reason, a case study was proposed to address HACCP certification in the Mexican and Chilean meat packers, in order to define opportunities for improvement.

Objects and methods

Field research was set as a case study of HACCP system implementation in two meat packers, one located in Mexico and the other in Chile. The study of these two packers was based on the proposal put forward by Flyvbjerg [12], based on a global context with the expectations and needs of the companies and under the requirement to place their products in international markets [13]. First, the main guidelines for the identification of the characteristics present in HACCP were identified [14], and prior to the study, the relevant requests were made to the management of each of the companies. The management information was collected during on-site stays for 43 and 14 days at the Mexican (ME) and Chilean (CE) meat packers, respectively, in 2018. Information gathered came from 15 indicators (Table 1) — five inherent in and ten external to the HACCP plan; the last ones had the potential to influence HACCP performance.

Table 1. Indicators inherent in and external to HACCP evaluated in the Mexican and Chilean meat packers

Indicators inherent in the HACCP plan
Process flow diagram
Product description
CCP identification
CCP monitoring
Verification of the system
Indicators external to the HACCP plan
System certification
Integrated management systems annexes
Legal authorizations
Current authorized in-process and export markets
Personnel age and education level
Sanitary performance standards
Prerequisites
Suppliers
Official inspection
Geographical conditions

Results and discussion

Indicators inherent in the HACCP plan

Meat packers' descriptions

General characteristics of each meat packer are shown in Table 2. The ME was under the Federal Inspection Type (TIF) certification, National Service for Agri-Food Health, Safety and Quality (Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria [SENASICA]). This certification guarantees strict quality and hygiene standards in plants, and thus the production of safe and high-quality products suitable for national and international markets. Activities are carried out in cattle slaughter, cutting, boning and vacuum packing. Around 98% of cattle slaughtered come from production units certified by Mexican federal authorities. The meat industry represents a link between consumers and agriculture and, therefore, requires an exhaustive analysis of the population's requirements and adjustment to raw materials of the stipulated quality [3]. Consequently, the implementation of HACCP represents an advantage in the whole phase of the production chain of meat products [15].

Table 2. General characteristics of the Mexican and Chilean meat packers

Characteristics	Mexican packer (ME)	Chilean packer (CE)
Altitude (masl)	60[1]	5,72[2]
Average annual temperature (°C)	31.7[1]	14.1[2]
Species slaughtered	Bovine	Swine
Certifications	Federal Inspection Type (TIF)	L.B. O'Higgins
Installed capacity (animals/shift)	400	2,000
Hours per shift	8	8
Cutting and boning rooms	1	1
Freezing capacity (t)	42	40
Refrigeration capacity (t)	450	160
Pallet store	1	1
Export quantities (t yr^{-1})	6,461.5	1,500
Fresh marketing (%)	80.7	0
Frozen marketing (%)	19.3	100
Accredited animal suppliers (%)	98	100
Number of employees	443	272

[1] [16] [2] [17]

The CE operation authorization came from the Health Service, L.B. O'Higgins, Warehouse-type storage: frozen food, refrigerated food by Regional Ministerial Health Secretariat (Secretarías Regionales Ministeriales de Salud SEREMI Salud) L.B. O'Higgins, and Agricultural and Livestock Service, L.B. O'Higgins.

Swine slaughtered come from farms certified by the agency Animal Production Units Program under Official Certification (Programa de Planteles Animales bajo Certificación Oficial [PABCO]), with compliance of the National Plan supervised by the Agriculture and Livestock Service (Servicio Agrícola Ganadero [SAG]), and the Chilean Trade Association of Swine Producers (Asociación Gremial de Productores de Cerdos de Chile [ASPROCER]), subjected to the official standards to monitor dioxins, furans and dL-PCB. The CE carries out swine slaughter and boning activities. The HACCP is certified, according to the requirements of the Recommended International Code of Practice, General Principles of Food Hygiene. Additionally, it places on the market refrigerated offal, bone-in and boneless by-products chilled and frozen. As stipulated in the HACCP concept, three stages in its design focus on evaluating the CCPs of any unacceptable risks. Finally, the identified control parameters are applied and followed up for prevention and reduction to acceptable levels [18].

Process flow

As indicated by Allata et al. [19], the operability of HACCP stands on seven principles aimed at maintaining control at acceptable levels of the identified hazards through the process flow chart. Then, the case study shows the primary and secondary activities of the process in the flow chart of both companies (Table 3). A sequential and unidirectional process flow was identified with on-site verification of the diagram as proposed by USDA [20]. Differences can be identified between the enterprises in terms of the design method and the mechanism used in the integration of raw materials. The ME applied a separate flow between the main flow diagram and the viscera flow diagram. However, both diagrams were operated under the same vertical format, in which the flow of operations was applied from top to bottom in an ordered list of process operations. In contrast, the CE used a single panoramic format, where both flows were processed together. It was presented in a single format, which can be viewed more accurately and quickly, thus facilitating operation. In addition, it presents both vertical and horizontal line recording and the various activities.

In both packers, the figures in the flowchart design were used correctly. However, there were differences between them, as the ME did not have different and precise pictogram in the description of the start and end activities in the flowchart. With this procedure, both companies claimed that the use of these diagrams led to better control of the relevant inputs and also facilitated the auditor in the interpretation. The ME flowchart had alternative diagrams in raw materials with a more significant number of structures and decision steps. However, the process of using abrasive chemicals for viscera blanching and leg peeling was not described.

The HACCP team was in charge of checking the flow diagrams on-site in order to make this management activity efficient. In this concept, it is indispensable to prepare the flow chart properly and ensure that it is thoroughly analyzed in the field, acquiring all the fundamental information. Therefore, having a flowchart with a structure that includes the required informative planning and detailed analysis leads to an integrated production process, which serves as a basis for the detection of possible deviations [21].

Product description

According to Pombo Marques [21], the authority of the HACCP team is represented by the team leader. The objective of this representative is to ensure the proper functioning of the HACCP plan. The leader's duties also include detailed monitoring to ensure compliance with legal criteria, reviewing all phases of the plan, attending to responsibilities and coordinating internal and external operations. In accordance with the HACCP plan of the packers of the study case, the team members carried out a concise and summarized description of the meat products. Table 4 shows the characteristics of both packers. The ME included chemical characteristics in greater detail, such as percentage of moisture, protein, fat and ash, while the CE omitted these elements. However, the latter fully described scalding by immersion and manual flaming. Although the ME performed spray sanitization, the description was

Table 3. Comparative flow chart of the Mexican and Chilean meat packers

Characteristics	Mexican diagram				Chilean diagram
	Process flow		Viscera flow		Complete flow
Main activities	41		16		35
Secondary activities	11		3		16
Decision stages	10		9		4
CCPs identified	3		1		2
Alternate Input Diagrams					
Number of times	2		1		1
Input	Water	Sanitizers	Chemicals	Packaging	Inks
Activities	2	4	6	3	4
Decision stages	2	2	3	1	0

Table 4. Comparative description of the products in the Mexican and Chilean packers.

Descriptive factors		Mexican enterprise (ME)	Chilean enterprise (CE)
Product family		Carcass meat	Rods
Components and ingredients		➢ Beef carcasses include the following tissues: muscle (the main one), connective, cartilaginous and adipose. ➢ Meat product, 100% beef.	➢ Swine carcasses may include tail, pillars, peripheral portion of the diaphragm, head, kidneys, feet and hide. ➢ By-products include heart, liver, kidneys, thymus, udder, blood, tongue, brain or fat of slaughter species; lungs are excepted from this category (RSA*, Title XI, paragraph I, article 269).
Packaging		No type of packaging is used for beef carcasses.	No type of carcass packaging is used
Shelf life (days)		7	12
Microbiological characteristics		➢ *E. coli* O157: H7 in 25 g — Absence ➢ *Salmonella* spp. in 25 g — Absence	The microbiological criteria established in accordance with national and international standards are stipulated in document FC-DC-HACCP-01- Plant Sampling Program.
Chemical composition (%)		Moisture 60 to 80	The description does not contain this type of information.
		Protein 16 to 25	
		Fat 2 to 10	
		Ash 1	
Organoleptic characteristics	Texture	It does not have this type of information	Firm and taut, elastic when raw and succulent when cooked.
	Odor	It does not have this type of information	Typical to slightly acidic pork. Without inappropriate odors (ammoniac or others).
	Color	It does not have this type of information	Pale pink, there may be variations in color in the same cut in the different muscles due to the amount of myoglobin with colors ranging from very pale pink to intense pink without representing alterations or pathologies in the quality of the meat.
Physicochemical characteristics		pH 5.5–6.5, a_w 0.99	Meat carcass pH 5.7–6.2, a_w = 0.985 Viscera pH 7, a_w = 0.985
Intended uses	Type of consumer	General public and industrial processes	General public
	Distribution conditions	Refrigerated (maximum 4 °C), clean vehicle, free of pests and in good physical condition.	The pork rods are cooled and then shipped by means of trucks conditioned with refrigeration equipment to maintain temperatures ≤ 7 °C.
	Sales locations	To distributors of meat in carcasses, cut and boned in the same establishment.	To distributors and points of sale, where they can be industrially roasted, or distributed nationwide, such as supermarkets or artisan butcher shops.
	Preparation methods	The consumer debones, cuts and cooks the product before consumption.	It is eaten cooked.
	Sensitive population	There is no sensitive population.	There is no sensitive population.
	Allergen declaration	No allergens are present in the finished product.	No allergens are present in the finished product.
Types of products		Beef in carcasses	Swine in carcasses
Storage and shipping temperature		The description provided does not include this type of information.	Rods: ≤ 7 °C (art 271)
			Fresh cuts: ≤ 7 °C (art 271)
			Fresh by-products: ≤ 3 °C (GB/T 20094–2006)
			Finished product: –2 to 2 °C.
Processes with microbicidal/ microbiostatic effect		The description provided does not include this type of information.	Scalding by immersion at 60 °C, manual flaming.

* Chilean Health Regulations for Food Products.
pH — Hydrogen potential.
a_w — Water activity in food.

not considered relevant, which is not in line with USDA recommendations [20]. The CE performed and described processes with microbicides and microstatics in blanching, while at the ME, the details of this information were not considered relevant.

Identification and monitoring of Critical Control Points (CCPs)

For the identification of CCPs, both packers applied the same methodology, which was based on the following activities: 1) establishment of criteria for hazard assessment; 2) identification of hazards; 3) identification of preventive measures; 4) identification of significant hazards; 5) hazard analysis; and 6) determination of CCPs using a decision tree. The CE HACCP team strictly applied the criteria of the Chilean Standard (NCh) 2861 of 2011, which interpretation is more feasible to the system auditor, and provides more reliable legal support. On the contrary, the ME evidenced no type of reference, which leads to more difficulty in its interpretation. Regarding the identification of hazards and preventive measures, both packers made similar procedure tables, where the possible hazards and the preventive measures implemented to prevent, eliminate or reduce the risk to an acceptable level were identified for each stage.

The identification of significant hazards was carried out in accordance with Chilean Standard NCh 2861, which implied having the result of an analysis of possible hazards. The occurrence had to be controlled in the operation stage to ensure safety. In the case of the ME, the procedure was similar but with some variants. In reference to the determination of CCPs, both companies operated the decision tree method. For each significant hazard identified, the result obtained was two CCPs for CE (Table 5), and three CCPs for ME (Table 6). Critical limits were established, and monitoring conditions were precisely specified. Both companies had a list of procedures and frequencies, at which activities were carried out in accordance with regulations [20]. ISO 22000:2018 [22] also indicates that at the end of the hazard assessment, it is necessary to select control measures, applying the CCP decision tree, in order to prevent or eliminate inherent food safety hazards and have a risk at an acceptable level.

As for good sanitary characteristics of carcasses, they are achieved by applying hygienic standards and a high level of sanitation during slaughter. Unacceptable results of microbiological analyses indicate sources of contamination and the lack of sanitary measures applied [23]. For example, a degree of fecal cross-contamination at the slaughterhouse is reflected in the prevalence of *Salmonella*-positive carcasses. Hygienic handling of the head and pluck during slaughter and dressing is of vital importance. Post-mortem inspection is necessary [24]. Besides, high counts of coliforms on carcass surfaces suggest a high incidence of fecal contamination, which means a potential risk for the consumer, and it reflects inadequate sanitation management during various activities, such as raw material handling, cleaning and sanitizing meat contact surfaces, and employee activities [25]. Most often, cross-contamination of carcasses occurs during such processing stages as skinning and evisceration as hides and the gastrointestinal tract are primary sources of pathogenic microorganisms [26]**.** Thus, strict sanitary and hygienic standards together with cold chain management must be applied in conjunction with the sanitary state of the refrigerated rooms. To minimize food safety problems, sanitary treatment of facilities is indispensable.

The cold processing step, such as rod cooling, represents an integral step in meat production, achieving stable quality and safety of these products. It is imperative to refrigerate them through a continuous cold chain in all phases of the technological process. Cold processing impacts the growth rate of microorganisms that lead to spoilage and also decreases the risk of pathogen growth [27]. Carcasses are typically cooled by circulating air in a refrigeration unit at a temperature of 0 to 4 °C, which is commonly applied in slaughterhouses for beef, pork, and other species. Initial chilling is the most important step in the cold chain to ensure appropriate food safety for carcasses [28]. According to Zhang et al. [29], rapid chilling achieves a significant reduction of bacteria on carcasses, followed by multi-step chilling. USDA-FSIS performs inspections of carcasses at all federally inspected slaughter facilities and verifies the compliance of establishments with food safety regulations [30].

Verification of the HACCP system

Verification is a strategy that ensures that the HACCP plan is working as intended according to the stipulated objective, methods, frequency and responsibilities. These activities generate evidence of its execution through records and documentation [31]. In the case of the CE, the HACCP team or part of it verified the system. The version of records and procedures was stipulated with three types of verification: daily, periodic and integral. The first was related to monitoring at each CCP and stipulating responsibilities and procedures. Periodic verification was carried out by the head of quality assurance with a monthly review of result records and microbiological updates of the system. Concurrently, the integral verification focused on the annual check of the operation of the entire HACCP system, which was performed by the whole team. In the ME, non-categorized verification was related, it was not systematized, and this information was not incorporated and consigned within the HACCP plan. The team met annually or whenever necessary in order to review the entire system. The categorization of the verification and frequencies included in the HACCP plan of the CE provided a comparative advantage since it provided greater clarity and organization. Critical limits were established, and monitoring conditions were precisely specified. Both companies had a list of procedures and frequencies, at which activities were carried out in accordance with regulations [20].

Table 5. Monitoring and follow-up of critical control points according to the HACCP plan in the Chilean packer.

HACCP plan	CCPs	Monitoring and tracking				
		What	Who	Where	How	When
Chilean enterprise	Inspection of finished rods CCP_{B1}	Presence of fecal contamination or gastrointestinal contents (visible)	Quality Assurance Monitor	Subsequent to operational step of the finished rod inspection	Visual Inspection: Exterior of legs; Interior and exterior of hindquarter; Interior and exterior of center quarter; Interior and exterior of forequarter; Hands; Interior and exterior of the head. The inspection includes opening of breast folds to check flare fat, ribs and diaphragm.	Every 45 minutes ± 10 minutes, from the beginning of the shift. PCC monitoring starts with the first 5 rods; the following PCC monitoring is performed randomly. To ensure randomness, once the inspection is finished, 2 rods are counted, and the third one is inspected until the 5 channels are completed, and so on every frequency until the end of the shift. The PCC monitoring will end with the last 5 rods.
	Rod cooling CCP_{B2}	Temperature and cooling time of rods	Dispatcher (rods)	In the cooling chambers in use.	The temperature of the rods (slaughter is performed during the immediately preceding shift), is monitored at the thermal center level.	At the end of the cooling process, the carcasses to be monitored are randomly defined according to the sample size which varies according to the batch size. The sample size is based on NChx. 44 of 2007.

Table 6. Monitoring and follow-up of critical control points according to the HACCP plan in the Mexican packer.

HACCP plan	CCPs	Monitoring and tracking				
		What	Who	Where	How	When
Mexican enterprise	Carcass inspection — zero tolerance CCP_{B1}	Presence of fecal contamination or gastrointestinal contents (visible).	Quality Control Supervisor	On hanging line, after the trimming stage	Visual inspection from the top of the leg, the entire hindquarter and forequarter of a carcass, up to the neck, is carried out at 360 degrees of a half carcass.	Frequency of 1 for every 10 channels, the channels to be monitored will be chosen by means of the random method (a draw is made prior to the start of slaughtering, where numbers from one to ten are recorded, and the number drawn is the number of the channel with which monitoring begins after all the channels have that digit as a base).
	Storage chamber temperature at 4 °C CCP_{B2}	Monitoring of the ambient temperature of the carcass chambers	Quality Control Supervisor	In the channel camera area	The monitoring start time for chambers 1 and 2 is 9 h. (+/– 10 min), for chambers 3, 4, 5 and 6 the time is 6 h. (+/– 10 min), and for chambers 7, 8 and 9 the time is 4 h. (+/– 10 min), by observing the thermometer located at one end of the chamber door frame on the sacrifice aisle side.	Maintenance shift personnel take and record the temperature every 2 hours (± 10 minutes), starting from the first measurement, additionally during the night and on weekends.
	Carcass sanitation CCP_{B3}	Sanitization of half carcasses	Quality Control Supervisor	Right after the vacuum steam intervention	The first carcass to be monitored is determined by selecting a number from 1 to 10; this choice is made at random before starting the process. The monitoring is done by directly observing the sanitization of half carcasses in their entirety, with a frequency of 1 out of every 40 carcasses; the time and consecutive number of the carcass is recorded.	Before starting the process and each time a dose of antimicrobial sanitizing solution is prepared.

Variables external to the HACCP plan

System certification, annexed integrated management systems

The directors of the HACCP team of both companies agreed that the choice of the certifying entity was made according to experience and support in the country, where the company operated at international level. The HACCP system at the CE was certified by Certification and Conformity Assessment (Certificación y Evaluación de la Conformidad LSQA S.A.), while for the ME, the certification was granted by the National Sanitation Foundation (NSF). In both cases, certification is renewed every year, as stipulated by the USDA guidelines [20]. Regarding the operation of annexed integrated management systems, the ME applied the Safe Quality Food Program (SQF) management system in conjunction with HACCP. On the other hand, the CE

operated only HACCP as a food safety and quality management system.

Legal authorizations

Both companies had all the mandatory authorizations at the national level, issued by the governmental regulatory bodies of each country. They also had permanent official inspections through the assignment of trained inspectors employed by government agencies, who were in charge of monitoring and controlling processes. The CE was authorized by governmental entities such as the Regional Ministerial Secretariat (Secretario Regional Ministerial [SEREMI]) of Health O'Higgins, the Sanitary Action Department and the Agricultural and Livestock Service, whose construction and operation are based on the Food Sanitary Regulations (Supreme Decree 977 of 1996 and Supreme Decree 94 of 2008). It had five current legal sanitary authorizations for the operation of the premises, meat and by-product processing, slaughter lines and holding chambers, animal deboning, product packaging, and the meat slaughter plant. In addition, it had also been approved by the National List of Livestock Product Exporting Establishments (Listado de Establecimientos de Productos Pecuarios [LEEPP]). On the other hand, the ME had two legal sanitary authorizations — one refers to the Federal Inspection Service (Tipo Inspección Federal [TIF]), awarded by SENASICA, and the other was Mexico Supreme Quality (Mexico Calidad Suprema), which is associated with the guarantee of agri-food products with high-quality standards.

Current authorized in-process and export markets

The managers defined the types of markets for export products in the two companies. For example, the enabled market was defined as a group of countries where the legal regulations and requirements had been complied with through visits by commissions made up of experts from the importing country and government regulatory delegates from the exporting country. However, regular exports had not yet been made. Markets in the process of being authorized referred to countries where exports were intended, but no legal authorization had yet been granted. Finally, formal export was stated for those markets, where there were actual records of exported products (Table 7). Regarding this last type of markets, the ME registered less than 28.5% of its production to the United States, Canada, Hong Kong and Japan, whilst the CE traded 77.3%. Another relevant aspect to note is the fact that the Chilean packer plant had more significant number of enabled markets, and a greater number of countries with formal exports. A notable aspect is the Russian market, which is considered one of the most discriminating and demanding markets. However, the CE has already exported approximately 30% of its production to this country.

Personnel age and education level

The ME had 443 regular employees between 30 to 40 years old. The CE had 272 employees between 20–40 years old; 88.6% were on regular permanent contracts, and 11.4% with time-limited contracts. The level of education varied according to the activities carried out. Concerning the personnel directly involved in the HACCP system and those directly related to technical operations, the monitoring and quality assurance operations were carried out by the quality team in both cases.

The employees' schooling was similar for both packers. The CE packer showed higher employee's schooling than the ME in the 1% employees channeled to quality control and monitoring activities. In addition, it should

Table 7. Comparison of markets and exports of the Mexican and Chilean packers

Mexican packer (ME)			Chilean packer (CE)		
Markets			Markets		
Enabled	*In-process*	*Formal export*	*Enabled*	*In-process*	*Formal export*
Puerto Rico	Korea	Canada	Russia	Salvador	Russia
Canada	Russia	United States	European Union	Colombia	European Union
United States		Hong Kong	Hong Kong		Hong Kong
Saudi Arabia		Japan	Japan		Japan
Hong Kong			South Korea		South Korea
Japan			Brazil		Brazil
Vietnam			Macao		Macao
Angola			Republic of Cabo Verde		Republic of Cabo Verde
Ghana			Dominican Republic		Dominican Republic
Gambia			Uruguay		Uruguay
Panama			Peru		
Qatar			Paraguay		
Egypt			Ecuador		
United Arab Emirates			Bolivia		
			Venezuela		

be noted that the quality team's level of education and years of experience varied significantly in each case. CE's personnel of the quality team were exclusively college graduated, with a minimum of three years of professional experience in food quality assurance system and HACCP training. These requirements were constantly being renewed. At the ME packer, quality team was made up of some technicians without experience directly related to quality activities. In contrast, all CE personnel were trained directly by professionals in this field, which represents a situation of more significant advantage for the company. The training of Mexican personnel in HACCP was taken externally by the team's management professionals, who in turn gave courses to the rest of the quality team once the packer hired them. This situation could be disadvantageous for the Mexican packer plant, considering the evaluation guidelines for the qualification of demanding markets.

There are reports that the role of employees is a significant factor in the operation of HACCP since knowledge and perception of the system are relevant. The function of the government is highlighted since it is indispensable for training and information campaigns for companies in the food sector [32]. Gehring et al. [33] pointed out that the level of training and supervision in the staff operation has an impact on the operability of HACCP. Therefore, the competencies and knowledge of food company employees have a direct relationship with positive achievements, and this highlights the imperative need for qualified and well-trained managers [34].

Sanitary performance standards

As regards the sanitary performance standards, both packers managed them in a similar way for drainage systems, ventilation, lighting, sanitary installations, integrated pest control, liquid and solid waste management, water quality, hygienic operations and facilities, equipment and utensils. Both carried out all corrective actions and contingency measures to comply with the sanitary regulations of each country. Likewise, they were executed in a similar way in terms of managing the personnel involved with job description procedures, staffing, obligations, and continuous training cycles on an annual basis.

Both companies had well-designed and paved access roads. The CE was located within the urban area, while the ME was outside the metropolitan area and required approximately 20 minutes to reach the facilities. Additionally, the CE had a mixed construction that consisted of fixed walls and panels, which can represent an advantage since it is feasible to make changes in the sizes of the areas without incurring exaggerated construction costs and delays. Furthermore, this type of construction facilitates the implementation of corrective actions and the adequate use of space without falling into the error of areas with tiny spaces that increase the risk of contamination or areas with large spaces that hinder the processes of cleaning, sanitization and maintenance of sanitary performance standards, which reduce an increase of hazards.

Prerequisites

These programs are indispensable prior to the implementation of HACCP since they are procedures that affect quality and safety of food and guarantee that the company has the basic operational conditions in place [35]. All documentation should have written records, and the HACCP team is responsible for evaluating the prerequisites [36]. In this study, both packers operated a number of prerequisites, which varied from one to the other. The ME had 20 programs in place, and the CE operated 24 programs. Three of them operated in general, and 21 fell into two categories, such as Standard Operating Procedures (SOPs) and SSOPs, with 15 and 6 programs, respectively. In regard to supplier management, both packers had an evaluation and follow-up program inherent in the prerequisites. The design of both programs was analogous since they considered fundamental aspects, such as acceptance and rejection criteria, required documentation and follow-up procedures.

Suppliers

As regards this case of study, the supplier management at both packers had evaluation and follow-up programs included in the prerequisites. The design of both programs was alike since they considered basic aspects such as acceptance and rejection criteria, required documentation and follow-up procedures. However, at the ME, other types of control procedures were based on the SQF (version 7.2), which prioritizes the control of suppliers under continuous improvement plans, food defense and environmental protection. It should be clarified that the CE packer did not implement a quality management system that strictly controlled its suppliers. According to the director of the quality assurance system, this was optional because the government regulates all suppliers. For this reason, it was sufficient to request current authorizations from suppliers and apply the basic procedures contained in the supplier program.

Meanwhile, the animal suppliers are regulated in both cases by the respective governmental entities. In Mexico, animals must belong to ranches accredited by the Ministry of Agriculture and Rural Development (Secretaría de Agricultura y Desarrollo Rural [SAGARPA]), while in Chile, they must come from farms under the Officially Certified Animal Stock Program (Programa de Planteles Animales bajo Certificación Oficial [PABCO]). It has been found that 98% of the animals slaughtered in the ME complied with this regulation, while 2% of animals were without any type of accreditation. In this case, the CE had a greater advantage in terms of trust and sanitary quality since 100% of the animals slaughtered had official certification. Prior to the HACCP implementation, the industry and the raw material suppliers involved in the program must identify the system's characteristics, and have the resources for the

initial and maintenance activities of the process [1]. Furthermore, raw material suppliers must have certifications generated by third parties [37].

Official inspection

Both packers had a permanent official inspection, which consisted of the assignment of trained inspectors employed by the government, to monitor and control the processes. The two packers had all the mandatory authorizations at the national level issued by the governmental regulatory agencies of each country. These activities were carried out by veterinarians, whom government agencies assigned according to the number of inspectors required by each packer. They applied each company's internal guidelines and analysis. In Mexico, SENASICA assigned four official veterinarians to inspect approximately 400 animals per day. In Chile, SAG ascribed seven similar professionals to carry out the inspection procedures of 2,000 animals per day. In this company, inspectors remained at the assigned inspection points during the entire slaughter day; that is, if there was not at least one official veterinary inspector at each inspection point, slaughter operations did not begin. In contrast, in the ME, antemortem and postmortem inspection was carried out intermittently.

Chilean guidelines stipulate that to be an Official Veterinary Doctor it is necessary to have at least one year of experience in similar positions in the public or private sector, have health compatible with the performance of the position, not be disqualified in any public position, have a professional degree in Veterinary Medicine granted by an institution of higher education recognized by the State and a certificate of courses. The required courses are veterinary epidemiology, veterinary medical inspection of slaughtered animals and meats, dictated by an entity recognized by the SAG, HACCP training, information for auditors, and management of the ISO 9001–2008 standard [38]. In contrast, SENASICA [39] requirements are summarized in personal documents, professional licenses, and proof of training in safety in the processes of production of meat goods, as issued by SAGARPA or academic institutions recognized by this secretariat. However, there are no specific requirements regarding professional experience. Therefore, qualified operators in food businesses are essential as they are necessarily subject to official controls and inspections both national and international in the case of exports [40].

Geographical conditions

Although the geographical conditions and location of the packers were heterogeneous, the sanitary status contemplated by the World Organization for Animal Health indicates explicitly that in terms of the diseases of interest for this case study, such as bovine spongiform encephalopathy, foot and mouth disease and classical swine fever, both Mexico and Chile have the recognitions of zones that are free of these diseases [41]. Mustafa [42] stated that geographic factors may constitute adverse factors, along with environmental, social and economic ones. For example, geographical conditions can constitute a threat to public health, like in a situation of lack of feasibility for workers and end users, and can become a drawback for crisis planning as a result of non-compliance with HACCP requirements. Geographical location is also a factor that influences compliance with GMPs protocols and GHPs because companies located in metropolitan areas are more easily accessible for evaluation due to the proximity to high officials and regulatory agencies, which leads to better levels of compliance [43]. In this case study, geographical conditions did not represent a relevant difference as both countries have achieved the same sanitary status.

It was evident in both enterprises that the implementation of comprehensive management systems attached to HACCP was essential for entering international markets. In fact, HACCP alone increases the probability that other countries will more readily accept foreign products with an increase in the export capacity of a company [44]. The HACCP enables exporting companies to design continuous improvement plans that contemplate adjustments not only in the plan but also in other annexed variables. The prioritization based on risk allows adequate optimization of available resources by management and quality control [45].

ME's HACCP plan did not have complete information in the product description as it omitted microbicidal and microstatic processes, which can lead to lower HACCP performance. Sotomayor and Silva [46] found that the lack of complete prerequisite programs is a barrier to HACCP implementation. On the other hand, the CE plan had more advantages in terms of straightforward interpretation of the process flow diagram, product description, CCP identification and system verification. In addition, the CE quality team had higher levels of education and experience than that of the ME. Lopez-Santiago et al. [47] established that technical barriers to HACCP performance include training and experience, among other things, which negatively affect the system.

The categorization of the prerequisites and the follow-up of the recommendations of the Codex Alimentarius and NCh 2861, carried out by the CE, contributed to optimizing the implementation of HACCP, facilitating the auditor's observation and analysis and increasing confidence thanks to the legal support of the system. Moreover, today, the mandatory nature of HACCP is contained and regulated in the health legislation of most countries [48]. Furthermore, the level of trust is strengthened by the fact that the total number of animals slaughtered by the CE came from the farms that were certified by the government control entity, which in turn demanded higher requirements from slaughterhouses and official inspectors, compared with the Mexican government entity. Currently, food production facilities are a subject of a wide range of research in terms of prevailing hygiene and sanitation. Although food safety constitutes compliance with various requirements in the different production links, more significant risks are con-

templated in the production of meat and meat products [26]. It is, therefore, essential to increase this type of case studies in companies that handle meat products in different parts of the world.

Conclusion

This article highlights how two packers, one Mexican (ME) and the other Chilean (CE), had the implantation of the HACCP system under national and international requirements. The ME showed an increase in the workload of the quality team without generating relevant competitive advantages for the enablement of international markets. The requirements demanded by the CE for personnel to join the quality team provided an advantage when evaluating qualifications in demanding markets such as the European Union. The quality team personnel of the CE had higher levels of training than that of the ME. In addition, the Chilean governmental control entity was stricter in the requirements for animal slaughterhouses and inspectors in charge of sanitary surveillance, which translated into greater confidence in the country's sanitary quality. Furthermore, all the animals slaughtered in the CE came from farms certified and accredited by the governmental entity, which increased the levels of confidence. On the contrary, this was not observed in the ME. Nowadays, exporting companies in developing countries are immersed in the context of quality and safety control of products. Therefore, it is essential to have quality standards such as the HACCP system certified and operated under the specified requirements. Finally, it is necessary to emphasize that the operability of the HACCP system complies with all the requirements stipulated for access and staying in high-income markets.

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IMPROVING THE FUNCTIONAL AND TECHNOLOGICAL PROPERTIES OF MINCED PORK USING A PROTEOLYTIC ENZYME

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Abstract
The use of pork obtained from intensively growing hybrid animals as the main raw material in meat processing, in particular sausage production, is accompanied by undesirable variability of consumer characteristics of sausage products and a decrease in the economic indicators of enterprise performance. The main technological disadvantages of processed pork are reduced water-binding capacity and significant loss of meat fluid, which in practice is usually compensated by the increased use of food additives and non-meat ingredients. The aim of the study was to assess the possibility of targeted improvement of the technological properties of minced pork using a proteolytic enzyme of animal origin. Model samples of minced meat were developed and cured for 24 hours at a temperature of 4 °C. The following control samples were used: minced meat with the addition of 2% edible salt and minced meat with the addition of 2% edible salt and 0.2% sodium bicarbonate. Moreover, 0.0001% chymotrypsin was added to the test samples containing similar curing ingredients. During electrophoretic study, in samples with the enzyme, an increase in low-molecular fractions (20 kDa, 15 kDa and lower) was observed, which indicated the manifestation of proteolytic activity of chymotrypsin in model systems. Enzymatic treatment led to an increase in pH and water-binding capacity. Cooking loss was reduced by 3 to 6 times, compared to Control 1. After cooking, histological studies of model systems showed that the test samples subjected to enzymatic treatment were characterized by a denser arrangement of structural elements, less pronounced cellular components of muscle tissue and the presence of glutin formed as a result of protein breakdown, filling the microcapillaries. Thus, the use of an enzyme preparation provides an opportunity for targeted improvement of the technological properties of pork obtained from intensively growing hybrid animals.

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Introduction

The use of proteolytic enzymes is one of biotechnological methods of raw material processing and has great potential in food production, including meat industry [1].

Currently, enzymes in meat technology are considered in two main applications. On the one hand, the action of endogenous tissue enzymes (endoenzymes) during meat aging after slaughter is being studied in order to create the best conditions and optimal duration for converting animal muscles into meat with high consumer value [2]. This transformation is of great importance for the meat industry. Due to endoenzymes, complex processes of "cell death" occur in meat, which form new functional and technological properties of muscle tissue, improving tenderness, juiciness, taste, flavor, color and texture of meat [3]. At the same time, a special role in this transformation is given to the multicatalytical proteinase complex consisting of calpains, lysosomal cathepsins and proteasomes [4]. On the other hand, an increasing number of studies are devoted to the use of exogenous proteases of non-meat origin, i. e. plant proteases [1,5,6], bacterial proteases [6,7] and fungal proteases [7], including new preparations whose properties and safety require careful study [8]. Unfortunately, in recent years, the interest of researchers in the use of proteolytic enzymes of animal origin (pepsin, trypsin, pancreatin, chymotrypsin), even as objects of comparison with enzymes obtained from other sources, has somewhat decreased.

Obtaining enzymes of animal origin is associated with the slaughter of animals. The meat industry has large resources of by-product raw materials [9], including those for the production of enzymes. The production and use of enzymes of animal origin simultaneously solves the problems of increasing the demand for low-value types of by-products, expanding the possibilities for their use, creating the additional value [10] and, consequently, reducing the environmental pressure from meat processing plants.

Recent studies show that the use of proteases in meat processing has the following main goals: improving the texture and tenderness of meat [5,6]; obtaining biologically active peptides, including from low-value by-products [10], reducing the allergenicity of introduced proteins or meat's own proteins in the manufacture of meat products [11,12]. At the same time, a completely justified goal of using proteolytic enzymes may also be improving the functional and technological properties of meat raw materials [13]. Currently, this goal is especially relevant for pork processing.

Pork is the most consumed meat in Asia and Europe, and in European countries up to 75% of pork is consumed in the form of processed meat products, which implies a high need for enterprises to stabilize the quality of this meat raw material [14]. At the same time, the widespread breeding of intensively growing hybrid animals in pig farming leads to undesirable variability in consumer characteristics of both raw materials and meat products, and is accompanied by a decrease in the economic indicators of enterprise performance.

The muscle tissue of intensively growing pigs is characterized by a high content of white muscle fibers capable of forming areas of "hypercontraction". This feature, which ensures intensive growth during the life of the animal, leads to the production of meat with reduced water-binding capacity, characterized by significant loss of meat fluid [15]. Subsequently, the processing of such meat is accompanied by a decrease in the yield of finished products. Pork obtained from intensively growing animals is characterized by such defects as PSE (pale, soft, exudative) and RSE (red soft exudative), as well as "destructured" meat [16]. In order to stabilize the quality and yield of sausages and other pork products, enterprises are forced to widely use non-meat ingredients and food additives in recipes [17]. However, the use of the latter causes constant concern among consumers about the composition and health benefits of such food products [18].

The main reason for the decrease in the functional and technological properties of pork is denaturation of muscle proteins [16,19]. For the production of high-quality finished products, the condition of muscle proteins (especially myofibrillar proteins), their ability to bind water and form new protein structures are of exceptional importance. In the presence of pork defects (PSE, RSE, destructured meat, muscle fiber damage), the ability of protein structures to bind water and interact decreases [16]. Their condition is close to the condition of proteins during thermal denaturation and may be aggravated by a decrease in salt in the product, a reduction in the curing duration, the absence of phosphates, protein oxidation and other factors. Already the initial stages of processing pork with quality defects are characterized by significant loss of water and salts dissolved in it, which affects the activity of endoenzymes in meat [16]. A decrease in the intensity of proteolysis as a result of protein denaturation may be an important reason for the insufficient quality of sausages and other finished products. However, excessive proteolysis of muscle proteins may also lead to excessive softness and an unpleasant taste of the meat product [13,16].

The study was based on the hypothesis of the possibility to use proteases during pork curing and minced meat formulation. In this regard, the choice of an enzyme of animal origin obtained from meat by-products seemed to be of the most interest. Thus, the purpose of this work was to assess the possibility of targeted improvement in the technological properties of pork obtained from intensively growing hybrid animals through the use of a proteolytic enzyme of animal origin, i. e. chymotrypsin, a preparation made in Russia.

Objects and methods

Research objects

The objects of the study were model systems based on minced meat made from pork with a mass fraction of adipose tissue of no more than 10%, treated and untreated with the enzyme, not subjected to heat treatment and after heat treatment.

To obtain the model systems, chilled pork was minced in a laboratory meat grinder through a grid with 5 mm hole diameter. Then the minced meat was weighed in portions of 500 ± 1 g followed by the introduction of pre-weighed and prepared food ingredients and additives, i. e. edible salt (extra grade boiled, Russol LLC, Russia), acidity regulator sodium bicarbonate (E500, $NaHCO_3$, Bashkir Soda Company JSC, Russia), chymotrypsin enzyme preparation (Samson-Med LLC, Russia). Then the minced meat was mixed to evenly distribute the components.

The pH value of pork before curing was 5.58 ± 0.02. According to literature [20,21], chymotrypsin effectively acts in an alkaline environment with a pH of 7.0 to 8.5 with an optimum at pH of 7.8 to 8.0. In this regard, to increase the pH value in the model system, the acidity regulator E500 was used.

The enzyme was pre-diluted with saline in the following ratio: 2 ml of saline per 0.01 g of the preparation. The enzyme was not added to the control samples, but the same amount of saline was added instead.

In total, two control samples and two test samples containing the following components in the composition of minced meat were prepared as model systems:

- Control 1–2% edible salt;
- Control 2–2% edible salt and 0.2% sodium bicarbonate;
- Test sample 1–2% edible salt and 0.0001% chymotrypsin;
- Test sample 2–2% edible salt, 0.2% sodium bicarbonate and 0.0001% chymotrypsin.

Model systems were stored in a refrigerator for 24 hours at a temperature of 4 ± 2 °C. After that, samples were taken for research of cured minced meat, including determination of protein fractional composition, as well as the functional and technological characteristics of minced meat, i. e. pH and water-binding capacity.

To obtain heat-treated model systems, samples of 100 ± 1 g were taken. The samples were packed in polyethylene-propylene bags on Webomatic Easy-pack vacuum packaging machine (Webomatic Maschinenfabrik GmbH, Germany) and subjected to heat treatment (cooking) in PE4310 laboratory water bath (Ekroskhim LLC, Russia) with a water temperature of 95 ± 1 °C. The cooking duration was 15 minutes. After cooking, losses during heat treatment were determined and the samples were sent for histological studies.

Research methods

The molecular weight distribution of protein fractions was analyzed by one-dimensional electrophoresis [22]. Sample preparation was performed as follows. 50 mg of each minced meat sample was taken and homogenized in 1000 µl of lysis solution (9 M urea (PanReac, Germany), 5% β-mercaptoethanol (PanReac, Germany), 2% Triton X-100 (Helicon, Russia), 2% ampholine pH 3–10 (Serva, Germany)) using Stegler S10 homogenizer (STEGLER, China). The resulting homogenate was clarified by centrifugation using Eppendorf 5427 R centrifuge (Eppendorf, Germany) at 14,000 rpm for 20 minutes. After that, 50 µl of the supernatant were collected in Eppendorf tubes and 50 µl of protein buffer (1 ml of 10% sodium dodecyl sulfate (SDS, PanReac, Spain), 250 µl of concentrated β-mercaptoethanol (PanReac, Germany), 625 µl of 0.5 M Tris-HCl (PanReac, Germany), 1.5 g of urea (PanReac, Germany) were added. Then bromophenol blue (Helicon, Russia) was added until a dark color and a volume was adjusted to 5 ml with distilled water. The resulting samples were heated in a boiling water bath for 5 minutes. For one-dimensional electrophoresis, VE-10 chamber (Helicon, Russia) filled with 12.5% polyacrylamide gel was used. Visualization and analysis of the images were carried out by staining proteins with Coomassie G-250 solution, consisting of 10% acetic acid (Komponent-Reaktiv; Russia), 25% isopropanol (PanReac, Germany), 0.05% Coomassie G-250 (Helicon, Russia). To remove unbound dye, 10% acetic acid (Komponent-Reaktiv, Russia) was used. Computer densitometry of the one-dimensional electropherogram, which was in a wet state, was performed using Bio-5000 Plus scanner (Serva, Germany) in 600 ppi 2D-RGB mode.

pH was measured by the potentiometric method using Testo 205 laboratory pH meter (Testo SE and Co., Germany).

The mass fraction of moisture was determined in accordance with GOST 9793–2016[1] by drying to a constant mass in a drying cabinet at a temperature of 103 ± 2 °C;

The water-binding capacity of the samples was determined by the Hamm and Grau method (pressing method) modified by Volovinskaya [23].

Cooking loss was determined by the gravimetric method: after heat treatment, the bags with samples were removed from the water bath and cooled to room temperature, then the liquid formed in the bag was drained, and a piece of minced meat was placed on filter paper to drain. Cooking loss in% was determined by the formula:

$$\text{Loss} = ((M_1 - M_2)/M_1) \times 100 \quad (1)$$

where M_1 is the weight of the sample before cooking;

M_2 is the weight of the sample after cooking and draining the liquid.

Histological studies were carried out in accordance with GOST 31796–2012[2] with sample fixation in accordance with GOST 31479–2012[3]. The pieces were placed in a 10% aqueous solution of neutral formalin and kept for 72 hours at room temperature. Then they were washed with cold running water for 12 hours. The washed material was first impregnated with a 12.5% gelatin solution, and then with a 25% gelatin solution in a thermostat at 37 °C for 12 and 24 hours, respectively. To make histological sections, pieces of 15 × 15 × 4 mm were cut from the fixed samples and placed in the freezer of MIKROM-NM525 microtome-cryostat (Carl Zeiss, Germany) for freezing to a temperature of minus 20 ± 3 °C. Frozen pieces were cut into 10 to 30 µm thick sections using a microtome knife. The sections were transferred to a glass slide and stained with Ehrlich's hematoxylin for 3 to 4 minutes. The sections were then rinsed with water to remove excess dye, immersed in a 1% hydrochloric acid solution until a pink color appeared, then in ammonia water until a blue color appeared, and rinsed again with water for 2 minutes. After this, the sections were stained with a 1% aqueous eosin solution for 1 minute and rinsed with water. The sections were then placed under cover slides. The prepared histological preparations were studied and photographed using AxioImaiger A1 light microscope (Carl Zeiss, Germany) with a magnification of x340 using AxioCam MRc 5 video camera. The images were processed using AxioVision 4.7.1.0 computer image analysis system adapted for histological studies.

Statistical analysis

All indicators were determined in 3 to 5 replicates. The obtained data were processed statistically with the determination of the mean value and deviation (M ± m). When pairwise comparing samples, the significance of differences was determined using the Student's t-test.

Results and discussion

After formulation (adding all components and mixing), the control samples and test samples did not differ in appearance, color, and texture (Figure 1).

[1] GOST 9793–2016 "Meat and meat products. Methods for determination of moisture content" Moscow: Standartinform, 2018. Retrieved from https://docs.cntd.ru/document/1200144231 Accessed April 16, 2024

[2] GOST 31796–2012 "Meat and meat products. Fast histological method of identification of composition structural components" Moscow: Standartinform, 2013. Retrieved from https://docs.cntd.ru/document/1200100067 Accessed April 16, 2024

[3] GOST 31479–2012 "Meat and meat products. Method of histological identification of composition" Moscow: Standartinform. Retrieved from https://docs.cntd.ru/document/1200097485 Accessed April 16, 2024

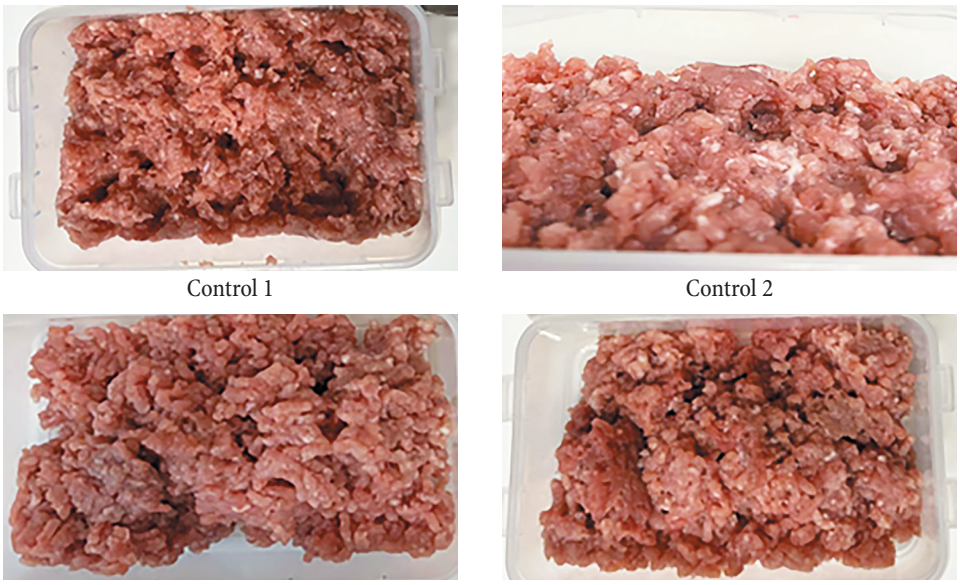


Figure 1. Appearance of model systems — control samples and test samples after formulation

In the test samples formulated with the addition of the enzyme preparation, a change in the texture of minced meat was observed after just 30 minutes. Minced meat became more viscous and stickier than in the control samples. Thus, the effect of chymotrypsin was observed already at the very beginning of the curing process. An increase in stickiness during the treatment of meat with proteases, e. g. proteases of microbial origin, was also noted by other authors [13].

Electrophoretic studies of the samples showed a change in the fractional composition of meat proteins as a result of exposure to the enzyme preparation (Figure 2).

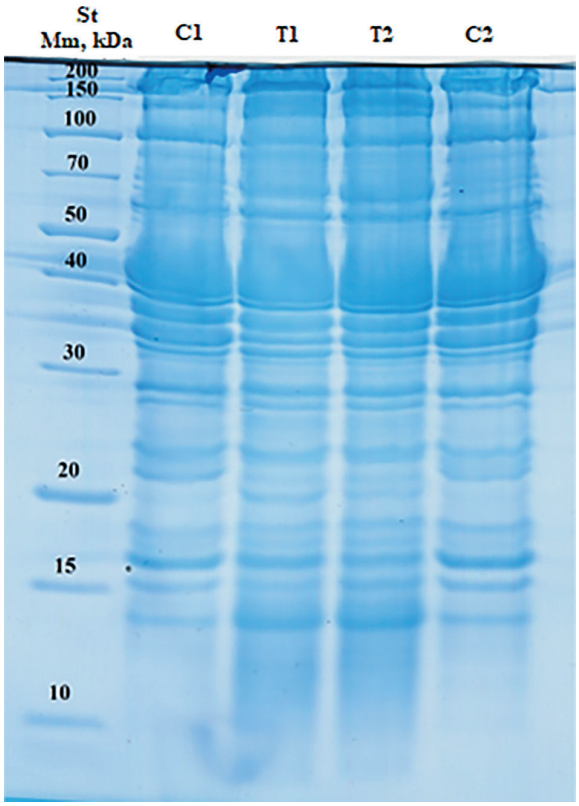


Figure 2. One-dimensional electropherogram of control samples and test samples. St — standard; C1 — Control 1; T1 — Test sample 1, T2 — Test sample 2, C2 — Control 2

Electropherogram at Figure 2 shows that the enzyme-treated Test sample 1 and Test sample 2 contained more low-molecular proteins with a molecular weight of 15 kDa and lower. Also, more intense bands on the electropherogram were observed in the region of about 20 kDa. On the contrary, in the region of 100 kDa, the intensity of protein bands in the test samples was reduced. This indicated the manifestation of the proteolytic activity of chymotrypsin in model systems, both without an acidity regulator (Test sample 1) and with an acidity regulator (Test sample 2), where the pH value of the system was increased.

The decrease in the manifestation of bands, as well as the presence of a protein background in the test samples, was due to the fact that the proteolytic activity of chymotrypsin degrades most protein fragments to low-molecular peptides. A similar formation of low-molecular peptides is observed during prolonged meat aging [24].

Among the decreased bands in the region from 30 to 50 kDa, there may presumably be protein fractions of myosin heavy chains (36.0 kDa) and actin heavy chains (41.7 kDa). The protein structure of myosin is sensitive to the action of chymotrypsin. During the degradation of myosin, degradation of the actin-myosin complex occurs simultaneously, which was noted in studies on the effect of enzymatic treatment on meat digestibility [25,26].

The study of pH, water-binding capacity (WBC) and cooking loss (Table 1) showed that control samples and test samples differed in functional and technological parameters.

Table 1. Functional and technological indicators of minced meat

Model systems (samples)	pH	WBC, %	Weight loss during cooking, % of initial weight
Control 1	5.79 ± 0.01^{b}	86.1 ± 3.7	12.2 ± 2.8^{b}
Control 2	6.05 ± 0.03^{a}	100.0 ± 0.0	4.1 ± 2.8^{a}
Test sample 1	5.83 ± 0.02^{ab}	100.0 ± 0.0	2.0 ± 1.4^{a}
Test sample 2	6.06 ± 0.07^{a}	100.0 ± 0.0	3.5 ± 0.7^{a}

Note: a — differences with Control 1 are statistically significant ($p < 0.05$), b — differences with Control 2 are statistically significant ($p < 0.05$)

The pH value in the control and test samples of minced meat after curing was higher than in the original meat raw material. The highest pH values were observed in Control 2 and Test sample 2, to both of which the acidity regulator was added. However, these samples did not have significant differences in pH.

In the model systems that did not contain the acidity regulator, the pH values were lower, but the differences between Control 1 and Test sample 1 were statistically significant ($p < 0.05$). This means that the action of the enzyme preparation contributed to an increase in the pH of minced meat.

Thus, the introduction of the enzyme preparation into the meat system during curing (Test sample 1) led to an insignificant increase in pH compared to the sample without the enzyme preparation (Control 1). However, the effect of the enzyme increasing the pH of minced meat was not observed compared to the addition of the acidity regulator.

After curing and aging for 24 hours, the WBC of three model systems, i. e. Test sample 1, Test sample 2 and Control 2, reached the maximum value of 100%. Cured meat did not release moisture during pressing for 10 minutes. Only Control 1 had a reduced WBC value of 86.1%. It should be noted that no differences were found in the mass fraction of moisture among the model systems. The mass fraction of moisture in the samples was 64.8 ± 3.7%.

The data obtained allowed to conclude that the introduction of the enzyme into the system led to an increase in the WBC of the cured meat to maximum values, as did the addition of the acidity regulator. This was consistent with the previously obtained results (using meat by-products), indicating that enzymatic treatment is accompanied by swelling of muscle fibers and an increase in WBC [13].

An important property of meat systems is their ability to retain moisture during heat treatment. Evaluation of loss during cooking of samples showed that the highest losses were typical for the Control 1 model system. In other samples, the losses during cooking were 3 to 6 times lower. This indicator also significantly differed in Control 1 compared to all other samples. This indicated that enzyme treatment and/or the introduction of an acidity regulator made it possible to reduce cooking loss.

Thus, the study of pH, WBC and cooking loss of model systems showed that enzymatic treatment with chymotrypsin combined with curing is able to improve the functional and technological properties of pork.

The results of the histological examination of the samples subjected to cooking showed the following.

The microstructure of Control 1 and Test sample 1 (Figure 3) was represented by fragments of muscle, connective and adipose tissues, and also contained a fine-grained protein mass formed as a result of mechanical destruction of raw meat. In Control 1, the muscle tissue fragments included non-swollen muscle fibers. The boundaries between them were well defined. The transverse striation of the fibers was clearly defined, the fiber nuclei were homogeneous. In Test sample 1, the muscle tissue fragments contained swollen round muscle fibers that were tightly adjacent to each other. The boundaries between them were poorly distinguishable, the transverse striation was not defined, the nuclei were shadow-like, and the swollen myofibrils were disintegrated.

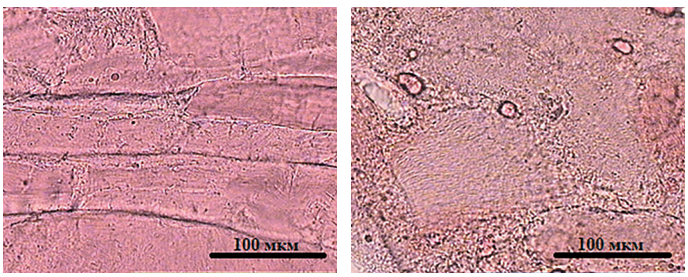
In Control 1, the average fiber diameter was 52.7 μm, while in Test sample 1 it was 75.0 μm. Test sample 1 differed from Control 1 in pronounced destructive changes in the form of multiple microcracks.

In Control 1, the connective tissue layers of the perimysium were characterized by dense bundles of collagen fibers, and the cell nuclei were clearly visible. In Test sample 1, the connective tissue layers of the perimysium were swollen and/or loosened, and the cell nuclei were poorly distinguishable.

In Control 1, the adipose tissue fragments included adipose cells containing fat droplets. The fat was also distributed in the form of 10–30 μm droplets in a fine-grained protein mass. The membranes of the adipose cells were not damaged. On the contrary, in Test sample 1, the membranes of the adipose cells were partly destroyed, due to which the fat was distributed in the fine-grained protein mass in the form of small 2–10 μm droplets.

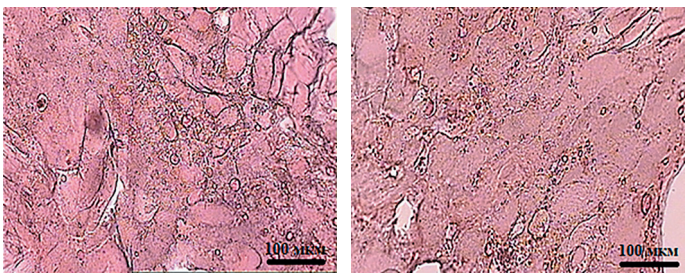
The microstructure of Control 2 was characterized by a denser arrangement of structural elements compared to Control 1. At the same time, as a result of the destruction of muscle fiber fragments to a fine-grained protein mass under the action of the enzyme preparation, Test sample 2 had even denser arrangement of meat structural elements (fragments of muscle, connective, and adipose tissue) (Figure 4).

In Control 2, the muscle fibers were swollen, the boundaries between them were clearly visible. The transverse striation of the muscle fibers that retained their integrity was expressed in most of the fibers. Destructive changes in the muscle fibers were in the form of transverse cracks with the formation of a fine-grained protein mass in the destruc-



a) b)

Figure 3. Muscle tissue fragments:
a) Control 1; b) Test sample 1. (Magnification × 340)



a) b)

Figure 4. Microstructure of the minced meat:
a) Control 2; b) Test sample 2. (Magnification × 340)

tion areas. In Test sample 2, more pronounced swelling of the muscle fibers was observed. In some areas, the fibers merged with each other, and the boundaries between them were not distinguishable. Transverse striation, on the contrary, was not expressed, the fiber nuclei were shadow-like, destructive changes in the form of multiple microcracks and transverse cracks were pronounced with the formation of a fine-grained protein mass in the destruction areas.

Different conditions of the sarcolemma of the muscle fibers were noted. In Control 2, the integrity of the sarcolemma was preserved, while in Test sample 2 it was damaged; in some areas, small fragments were destroyed to a fine-grained protein mass, which enhanced the interconnection of the structural elements that had preserved their integrity.

It was noted that in Test sample 2 sample, the preserved muscle fibers were also more tightly adjacent to each other. The average diameter of muscle fibers in this sample was 88.0 μm, while in Control 2 it was 73.8 μm.

In Control 2, swollen or partly loosened connective tissue layers of the perimysium were observed. In some areas, a mass of glutin was formed, filling the microcapillaries. In Test sample 2, the loosening of the connective tissue layers and the formation of a homogeneous mass of glutin was more pronounced. Glutin was located in the microcapillaries in the form of a homogeneous structureless mass stained with basic dyes.

Differences in the condition of adipose cells were also noted. In Control 2, the adipose cell membranes were not damaged, and the fat was distributed in the fine-grained protein mass in the form of small 5–10 μm droplets. In contrast, in the Test sample 2, the adipose cell membranes were destroyed, and the fat was distributed in the form of 1–3 μm droplets in the fine-grained protein mass.

Thus, the test samples treated with the enzyme were characterized by a denser arrangement of structural elements, a less pronounced cellular components of muscle tissue, and the presence of glutin, a nitrogenous gelatinous substance that filled the microcapillaries and was formed as a result of protein degradation.

In our study, chymotrypsin was selected as the enzyme preparation. Chymotrypsin is not included in the list of enzyme preparations permitted according to TR CU029/2012 "Safety requirements for food additives, flavorings and processing aids". However, the current international practice of assessing the safety of enzyme preparations shows that pancreatic enzymes do not raise concerns about their safety under the expected conditions of use for food purposes on the basis that they originate from edible parts of animals [27].

Chymotrypsin is a serine protease (endopeptidase) and is found in the pancreas of animals. Chymotrypsin has one polypeptide chain of 245 amino acid residues and a molecular weight of 25.7 kDa [28]. This enzyme is currently considered one of the most significant proteolytic enzymes, which is widely used in the food industry and medicine [29]. The mechanism of chymotrypsin action is that it acts on a non-reactive carbonyl (–C=O) using a nucleophile. This enzyme does not exhibit allosteric effects, i. e. it does not have an active center that affects the conformational state of the enzyme [30]. Chymotrypsin exhibits its specificity by catalyzing the hydrolysis of peptide bonds at the C-terminal side of tryptophan, tyrosine, phenylalanine and leucine (the latter to a lesser extent), releasing polypeptides. In foreign practice, chymotrypsin is permitted in protein processing to obtain hydrolysates for use as ingredients in formulas for infants and young children [27].

All the results obtained, i. e. electrophoresis data, functional and technological indicators and histological examination data, confirmed the positive effect of enzymatic treatment. Previous studies also showed that treatment with proteases led to an improvement in the functional and technological characteristics of pork [13]. This result is consistent and may be explained by the fact that water holding capacity by myofibrils improves after enzymatic cleavage of denatured protein structures due to the formation of new hydrophilic centers and a change in the charge of molecules [31]. However, there is another opinion that denatured proteins, in particular sarcoplasmic proteins, contribute to the retention of moisture in muscle tissue [32], which may cast a doubt on the effectiveness of pork enzymatic treatment. Nevertheless, the most important functional proteins of meat are myofibrillar proteins, accordingly, their condition and transformation are most responsible for the quality of the final product [33].

In our study, parallel control samples and test samples were presented, differing only in the presence an acidity regulator. Analysis of the results showed that the presence of E500 food additive in the model systems was not mandatory. The use of enzyme treatment made it possible to achieve the same technological effect as the introduction of an acidity regulator. This clearly confirms the validity of the opinion by a number of authors that the use of proteases as an environmentally friendly material has not only economic advantages, but also far-reaching positive consequences in achieving sustainability [29].

We also noted that the desired technological effect on improving the functional properties of minced pork was achieved at an aging temperature of 4±2 °C (corresponds to the temperature conditions in the meat curing chamber), although most exogenously used proteolytic enzymes of plant origin have an optimal temperature of 50 to 70 °C, which corresponds to the temperatures of heat treatment of meat products [4]. With regard to chymotrypsin, there is evidence that its optimal activity begins at 30 °C [27]. Our results show that enzymatic treatment of meat with chymotrypsin can be easily integrated into pork processing.

However, other conditions and other proteolytic enzymes may obviously be selected for specific technological solutions. Many authors studying various preparations emphasize the importance of the functional state of proteins and the positive role of enzymes in achieving the required quality indicators [4,26,34]. Thus, it was reported that papain treatment had a positive effect on the functional,

rheological and physicochemical properties of myofibrillar proteins. Compared with the control samples, the fermented samples of myofibrillar proteins showed better functionality. Moreover, papain treatment led to an increase in hydrophobic groups on the surface of proteins and a decrease in the number of α-helix and β-sheet structures, which contributed to a change in the conformation of proteins, improving their solubility and emulsifying properties [34].

Thus, in the future, the use of proteolytic enzymes in the production of meat products may be considered in the context of creating new effective technological approaches to improve the functional and technological properties of meat raw materials.

Conclusion

The results of the studies on model systems showed that the minced pork treatment with the enzyme preparation improves its functional and technological properties, contributes to an increase in WBC, reduces the product weight loss during cooking, and increases the density of the microstructural components of minced meat. At the same time, the improvement of the functional and technological properties of pork is accompanied by a partial degradation of muscle proteins and formation of low-molecular protein fractions of 20 kDa and below. The results obtained convincingly proved the targeted improvement of the technological properties of pork obtained from intensively growing hybrid animals through the use of a proteolytic enzyme of animal origin. Nevertheless, for the practical use of proteases in industrial pork processing, further studies are needed to select the most economically acceptable enzyme preparation, as well as to determine the optimal duration and conditions for enzyme treatment, including depending on the technology and type of the final product.

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PHYSICOCHEMICAL, MICROBIOLOGICAL, AND ORGANOLEPTIC PROPERTIES OF FERMENTED LAMB SAUSAGE ENHANCED BY JACK BEAN FORTIFICATION

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Abstract
Processing lamb meat into fermented sausages can reduce the risk of spoilage and extend shelf life. Fermented sausages are commonly made using lactic acid bacteria (LAB), resulting in a product that is acidic and less firm. Therefore, it is necessary to add jack bean flour as a binding agent. This study aims to analyze physicochemical, microbiological and organoleptic characteristics of premium IPB lamb fermented sausages with varying proportions of jack bean flour (0% and 30%). The analyses included pH, water activity (a_w), total acid, water, ash, fat, crude protein, carbohydrate, total LAB, E. coli, S. aureus, texture profile analysis (TPA), and sensory evaluation. The incorporation of jack bean flour into lamb meat fermented sausages can elevate the pH, springiness, chewiness, crude protein, and carbohydrate content of the fermented sausages. Furthermore, the addition of jack bean flour may reduce the total acid, a_w, gumminess, water content, and bacteria (E. coli and S. aureus). The sensory aspects such as color in fermented sausage with addition of jack bean flour were preferred by the panelists. The addition of jack bean flour increased the total unsaturated fatty acids and essential amino acids in fermented sausages. The incorporation of jack bean flour aligns with the Indonesian National Standards for Meat Sausages 3820–2015 concerning moisture, fat, and protein content. This study suggests that incorporating lamb fermented sausage with 30% jack bean flour could result in significant benefits, including increased nutrition, enhanced sensory quality, improved texture, and extended storage life for fresh lamb products.

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Introduction

Meat is a crucial component in meeting nutritional needs due to its complete and balanced proteins, essential amino acids, as well as various minerals and vitamins [1]. Lamb meat is one of the most widely consumed meats in Indonesia. The average protein content in sheep meat is 9.65%, fat content is 20.59%, water holding capacity (WHC) is 24.6% and cholesterol content is 19.2 mg/100 g of meat [2]. The high water and nutrient content in lamb meat make it susceptible to quality deterioration or spoilage (*perishable food*) [3]. The deterioration of quality in lamb meat can be caused by physical, chemical, and biological contaminants [4]. The processing of animal-origin food ingredients can suppress or inhibit the growth of bacteria in food products, extending the shelf life of meat, preventing spoilage, enhancing digestibility, and diversifying processed meat products. Among processed lamb meat products are fermented sausages. Fermented sausages are food products obtained from a mixture of meat, fat, spices, or seasonings, with or without the addition of lactic acid bacteria (LAB) as starter cultures, which are then stuffed into sausage casings [5].

The acid content produced by LAB can inhibit the growth of pathogenic bacteria and food spoilage bacteria, making LAB a group of beneficial bacteria that meet the GRAS (Generally Recognized as Safe) status, which means that they are safe for humans and can be applied as probiotic agents [6]. Lactic acid bacteria (LAB) as a source of probiotics have functional properties such as antihypertensive, antimicrobial, antidiabetic, antioxidant, and anticancer effects [7,8,9]. Additionally, according to Beltrán-Barrientos et al. [10], probiotics can also boost the immune system and inhibit the activity of cholesterol-forming enzymes, thereby reducing cholesterol levels in the body. Other functional properties include hypocholesterolemic effects and the production of bioactive peptides. In the production of fermented sausages, LAB play a crucial role in converting carbohydrates into lactic acid. One widely used and commercially available culture is *Lactobacillus plantarum* [11].

Typically, fermented sausages are made using only LAB, resulting in a less solid texture and an acidic taste [12]. These sausages can be enhanced by incorporating binding agents such as jack bean flour. In Indonesia, jack bean yields are approximately 3.9–4.6 tons per hectare, compared to soybeans at 1.7–2.6 tons per hectare [13]. Jack beans contain 27.4% protein, 66.1% carbohydrates, and 2.9% fat, being a good source of carbon and nitrogen for bacterial growth [14]. They can be processed into flour for using in various products including cakes, cookies, crackers, nuggets, tempeh, and tofu, and can also be used in fermented foods such as soy sauce and tauco. However, the use of jack bean flour in sausages is limited due to toxic substances, such as cyanide, phytic acid, tannins, saponins, and oxalates, which can cause undesirable tastes and toxicity [15]. These levels can be reduced through soaking, peeling, boiling, cooking, and fermentation. Recommended phytic acid consumption is 25 mg/100 g [16]. Using jack beans as a binding agent in fermented sausages can reduce the dependence on imported tapioca and soy flour in Indonesia. This study aims to analyze physicochemical, microbiological and organoleptic characteristics of premium IPB lamb fermented sausages with varying proportions of jack bean flour (0% and 30%).

Materials and Methods

Materials

Premium IPB lamb meat was obtained from farms in Banjarnegara, Central Java. Premium IPB lambs were slaughtered at PT Pramana Pangan Utama, IPB University. The entire process in the research complied with the "Institutional Animal Care and Use Committee (IACUC)" issued by IPB University (approval ID: 118–2018 IPB). Jack beans (*Canavalia ensiformis*) were obtained from East Java. The probiotic strain *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 was taken from the collection of the Laboratory of Animal Product Technology, IPB University.

Production of jack bean flour

Five kilograms of jack bean seeds were soaked in water for 72 hours with water changes every 12 hours, then peeled and cleaned. The seeds were dried in an oven at 60 °C for 7 hours. Once thoroughly dried, the jack beans were finely ground and sifted through an 80-mesh screen [17].

`Preparation of starter culture

The *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 culture was refreshed by incubating it in 9 mL of de Man, Rogosa and Sharpe Broth (MRS Broth) medium at 37 °C for 24 hours until turbidity indicated adaptation. This refreshed culture was then inoculated at 2% into the sterile 10% skim milk solution and incubated at 37 °C for 24 hours to form the mother culture. Subsequently, intermediate and working cultures were produced through successive steps. The initial population of the working culture was determined by inoculating it onto de Man, Rogosa and Sharpe Agar (MRS Agar) medium with a suitable culture having over 10^8 CFU mL^{-1} [18].

Production of fermented sausages

The production of fermented sausages began with standardizing 80% of meat, separating whole meat from meat containing fat. The standardized meat underwent grinding, freezing, and mixing with seasoning ingredients including salt (2%), sugar (0.5%), garlic powder (1.5%), pepper (0.5%), nutmeg (0.3%), jack bean flour (0% and 30%), and lactic acid bacteria (5%). The mixture was then filled into sausage casings and conditioned at 27 °C for 24 hours. After resting, the sausages underwent cold smoking for 5 hours over 2 days at 28–30 °C. Finally, the fermented sausages were ready for testing [19].

Analysis of physicochemical characteristics

The physicochemical analysis of fermented sausage samples encompassed the measurement of pH [20], water activity (a_w) [20], total acid [21], water content [20], ash content [20], fat content [20], crude protein content [20], carbohydrate content [20], total amino acids [22], total fatty acids [23], and texture profile analysis (TPA) [24].

Analysis of microbiological characteristics

The microbiological analysis of fermented sausages involved assessing total LAB, *E. coli*, and *S. aureus*. Samples of 25 g fermented sausages were diluted in the Buffered Peptone Water solution to achieve dilutions ranging from 10^1 to 10^8. Colony counting was conducted by inoculating dilutions onto specific agar media for each bacteria type: de Man, Rogosa and Sharpe Agar for LAB, Eosin Methylene Blue Agar for *E. coli*, and Baird Parker Agar Base for *S. aureus*. The dishes were then incubated at 37 °C for 48 hours [25].

Analysis of organoleptic characteristics

Organoleptic testing of fermented sausages utilized both hedonic and hedonic quality tests. Variables assessed included color, taste, aroma, and texture with 35 panelists rating each on a scale from 1 to 4 [26].

Data Analysis

The research data were analyzed using T-test and Kruskal-Wallis test for sensory analysis. The applied treatments were as follows:

P0: Fermented lamb sausage and *Lactobacillus plantarum* IIA-1A5

P1: Fermented lamb sausage and *Lactobacillus plantarum* IIA-1A5 with the addition of 30% jack bean flour.

Results and discussion

In this study, samples of fermented sausages underwent characteristic analyses, including determination of pH, water activity (a_w), total acid, water content, ash content, fat content, crude protein content, carbohydrate content, texture analysis, total LAB analysis, analysis of *Escherichia coli* and *Staphylococcus aureus*. The results of these tests are presented in Table 1.

Tabel 1. Characteristic of fermented sausage

Parameter	Treatment		Description
	Control	With jack bean flour	
Physicochemical			
pH	4.50 ± 0.02[a]	5.42 ± 0.02[b]	—
Total acid (%)	1.70 ± 0.06[a]	1.24 ± 0.05[b]	—
a_w	0.85 ± 0.00[a]	0.82 ± 0.00[b]	—
Springiness (mm)	1.17 ± 0.17a	1.87 ± 1.25[b]	—
Cohesiveness	0.86 ± 0.06	0.78 ± 0.05	—
Gumminess (N)	2211.76 ± 140.38[a]	1998.50 ± 142.55[b]	—
Chewiness (N)	3959.93 ± 516.17[a]	5527.45 ± 552.46[b]	—
Proximate composition			
Water content (%)	62.52 ± 1.14[a]	47.27 ± 1.24[b]	Max. 67
Ash content (%)	4.14 ± 0.05	4.28 ± 0.09	Max. 3
Fat content (%)	5.07 ± 0.70	5.26 ± 1.18	Max. 20
Crude protein content (%)	26.11 ± 0.27[a]	27.32 ± 0.82[b]	Min. 13
Carbohydrate (%)	2.16 ± 0.75[a]	15.87 ± 1.45[b]	Max. 8
Microbiology			
Lactic acid bacteria (log CFU/g)	7.09 ± 0.09	7.01 ± 0.06	Dough
	7.20 ± 0.09	7.12 ± 0.01	Conditioning
	8.14 ± 0.02	8.13 ± 0.01	Smoking 1
	8.19 ± 0.04	8.16 ± 0.01	Fermented Sausage
Escherichia coli (log CFU/g)	1.73 ± 0.41[a]	0.87 ± 0.10[b]	Dough
	—	—	Fermented Sausage
Staphylococcus aureus (log CFU/g)	1.20 ± 0.09[a]	0.91 ± 0.01[b]	Dough
	—	—	Fermented Sausage

Note: Different letters in the same row indicate significant differences (P < 0.05).

The pH values of fermented sausages with the addition of jack bean flour were higher compared to those without the addition. This can be attributed to the fact that jack bean flour has a neutral pH of 7 [27]. The addition of jack bean flour is expected to make sausages more palatable to consumers due to its less acidic taste. A decrease in pH can be attributed to the growth of LAB that produces lactic acid [19]. Total acid value of fermented sausages with the addition of jack bean flour was lower compared to those without jack bean flour. The addition of jack bean flour can increase the pH in the fermented sausage product, thereby reducing the total acid. Total acid is influenced by the fermentation process in the product, occurring over three days. Total acid values are inversely proportional to the pH values produced; as pH decreases, total acidity increases [19].

The a_w value of fermented sausages with the addition of jack bean flour was lower compared to those without jack bean flour. The hygroscopic nature of jack bean flour allows it to absorb water effectively, reducing free water content [28]. This property depends on protein quality and the presence of polar amino acids [29]. Adding jack bean flour to fermented sausages increases the polar amino acid composition, lowering their water activity [29]. Lower a_w values significantly enhance food safety as they can inhibit the growth of pathogenic bacteria within the product [19].

Springiness and chewiness increase in fermented sausages with jack bean flour due to its high amylopectin content [30]. The elevated amylopectin contributes to elasticity, while glutamic acid in jack beans enhances water absorption, resulting in a denser texture [31]. This aligns with studies indicating that high amylopectin content produces denser and more elastic products [32]. Gumminess decreases in sausages with jack bean flour due to its water-absorbing properties [31]. The addition of jack bean flour did not affect cohesiveness of sausages.

The addition of jack bean flour decreases the water content in fermented sausages due to its hygroscopic properties, as it can effectively absorb water [28]. Glutamic acid, the predominant amino acid in jack bean flour, contributes to this water absorption [31]. Consequently, sausages made with jack bean flour have a denser texture. The inclusion of jack bean flour did not affect the ash and fat content of the sausages. However, it enhanced both the protein and carbohydrate content, suggesting improved nutritional value. Jack bean flour is rich in protein and has a low glycemic index, making it a promising functional food ingredient [33,34,35].

The addition of jack bean flour to fermented sausages did not significantly affect the total LAB count, as shown in Table 1. However, sausages without jack bean flour tended to have slightly higher LAB counts, possibly due to variations in sausage weight and smoking process conditions. LAB thrive within a pH range of 3.5–10.0 and temperatures of 5–45 °C [36]. Fermented sausages with jack bean flour exhibited lower levels of *E. coli* and *S. aureus* bacteria, likely due to antimicrobial compounds present in jack bean flour, such as flavonoids [37]. Additionally, LAB starter cultures can contribute to reducing *E. coli* populations due

to their antibacterial properties [19]. For example, *L. plantarum* IIA-1A5 has been reported to possess antibacterial properties against pathogenic bacteria, including *E. coli, S. aureus* [38] and *Salmonella typhimurium* [39].

Table 2. Fatty acid characteristics of fermented sausage

Parameter	Treatment (% w/w)	
	Control	With jack bean flour
Total saturated fatty acids (SFAs)		
Butyric acid C4:0	2.71	0.07
Caproic acid C6:0	0.02	2.19
Caprylic acid C8:0	0.05	0.01
Capric acid C10:0	0.09	0.07
Lauric acid C12:0	0.07	0.07
Myristic acid C14:0	1.23	1.18
Pentadecanoic acid C15:0	0.35	0.35
Palmitic acid C16:0	11.36	11.00
Heptadecanoic acid C17:0	0.71	0.69
Stearic acid C18:0	7.46	7.15
Total poly-unsaturated fatty acids (PUFAs)		
Linolenic acid C18:3n3	0.08	0.76
Linolelaidic acid C18:2n9t	0.16	0.19
Total mono-unsaturated fatty acids (MUFAs)		
Myristoleic acid C14:1	0.05	0.05
Palmitoleic acid C16:1	1.02	1.09
Oleic acid C18:1n9c	19.13	21.07
Elaidic acid C18:1n9t	1.86	1.95
Total fatty acids	46.36 ± 11.44	47.90 ± 11.88
Total saturated fatty acids (SFAs)	24.05 ± 1.09	22.78 ± 0.90
Total unsaturated fatty acids (UFAs)	22.84 ± 7.58	25.11 ± 8.30
Total poly-unsaturated fatty acids (PUFAs)	0.24 ± 0.05	0.95 ± 0.40
Total mono-unsaturated fatty acids (MUFAs)	22.06 ± 9.10	24.16 ± 10.05
Ratio of total SFAs/UFAs	1.05	0.90

The content of total fatty acids, saturated fatty acids, and unsaturated fatty acids in premium lamb meat is 73.35%, 39.64%, and 30.37%, respectively. The content of unsaturated fatty acids, such as oleic acid, palmitoleic acid, and linoleic acid, is 24.26%, 1.58%, and 2.37% [40]. Table 2 shows that adding jack bean flour reduces butyric acid and increases caproic acid levels in fermented sausages due to the fermentation process converting butyric acid into other compounds [41]. According to Kinteki at al. [42], increased caproic acid levels are a byproduct of proteins and amino acids produced by LAB. Sridhar and Sharma [43] state that jack beans contain omega-6 fatty acids, including linoleic acid, linolenic acid, cis-11,14-eicosadienoic acid, and arachidic acid, and omega-3 fatty acids such as timnodonic acid, docosahexaenoic acid, and docosapentaenoic acid. Therefore, adding jack bean flour can increase the linolenic acid and oleic acid content in fermented sausages, enhance unsaturated fatty acids, and reduce the SFAs/UFAs ratio. A lower SFAs/UFAs ratio improves texture and flavor stability, making the sausages softer with a more complex taste. Overall, the fatty acid profile of fermented sausages with jack bean flour is better than that of control sausages, offering health benefits such as reduced cardiovascular disease risk and enhanced product flavor [44].

Table 3. Amino acid characteristics of fermented sausage

Parameter	Treatment (%w/w)	
	Control	With jack bean flour
Essential amino acids		
Lysine	1.80	1.77
Leucine	1.69	1.78
Isoleucine	0.92	0.98
Methionine	0.44	0.36
Threonine	0.90	0.98
Phenylalanine	1.43	1.42
Valine	0.99	1.03
Histidine	0.89	1.21
Non-essential amino acids		
Alanine	1.33	1.19
Aspartate	1.96	2.11
Glutamate	3.59	3.42
Arginine	1.05	1.14
Glycine	1.26	0.97
Tyrosine	0.62	0.62
Serine	0.79	0.90
Total amino acids	19.67 ± 4.64	19.87 ± 4.69
Essential amino acids	9.06 ± 0.46	9.53 ± 0.47
Non-essential amino acids	10.60 ± 1.01	10.35 ± 0.97

Table 4. Chemical score of amino acids

Essential amino acids	Treatment	
	Control	With jack bean flour
Isoleucine	57.50	61.25
Leucine	34.50	36.32
Lysine	59.50	58.51
Methionine + cysteine	35.92	29.40
Phenylalanine+ tyrosine	56.94	56.66
Threonine	56.25	61.25
Tryptophan	—	—
Valine	39.60	41.20

Table 3 shows that the essential amino acid content in fermented lamb sausages with the addition of jack bean flour is higher compared to fermented lamb sausages without jack bean flour. According to Kanetro et al. [45], jack bean flour contains amino acids such as aspartic acid (2.41%), glutamic acid (2.10%), asparagine (0.71%), histidine and L-serine (1.49%), threonine (2.21%), glutamine (1.01%), arginine (1.41%), tyrosine (0.94%), lysine (3.00%), alanine (1.01%), glycine (1.01%), tryptophan and methionine (0.94%), valine (1.00%), phenylalanine (1.97%), isoleucine (1.01%), and leucine (1.09%). The histidine and serine content in jack bean flour also increases the histidine and serine content in fermented sausages with the addition of jack bean flour. Products with high essential amino acid content are considered better because essential amino acids cannot be produced by the body and must be obtained

from dietary sources. High levels of essential amino acids are beneficial for health as they are crucial for growth, repair, and maintenance of body tissues [46]. Table 4 displays the chemical score of amino acid values. Further examination revealed that all samples lack tryptophan. Additionally, sausages with added jack bean flour showed higher scores for isoleucine, threonine, and valine. These elevated scores indicate sufficient or even excess levels of these essential amino acids, crucial for various body functions. Essential amino acids, indispensable for human health, must be obtained through diet. Hence, higher chemical scores for these amino acids signify a superior amino acid profile in supporting overall body functions [47].

Table 5. Organoleptic characteristics in fermented sausage

Parameter	Treatment	
	Control	With jack bean flour
Hedonic test		
Color	2.25 ± 0.44[a]	2.00 ± 0.48[b]
Taste	2.25 ± 0.57[a]	2.86 ± 0.79[b]
Aroma	1.84 ± 0.52	1.95 ± 0.57
Texture	2.02 ± 0.55[a]	2.34 ± 0.64[b]
Hedonic quality test		
Color	1.07 ± 0.25[a]	2.02 ± 0.34[b]
Taste	2.68 ± 0.47[a]	2.40 ± 0.58[b]
Aroma	2.75 ± 0.44[a]	2.97 ± 0.55b
Texture	2.20 ± 0.41[a]	1.43 ± 0.50[b]

Note: Different letters in the same row indicate significant differences (P < 0.05). Hedonic scale: 1 (like very much), 2 (like), 3 (dislike), and 4 (dislike very much). Hedonic quality scale: Color: 1 (dark brown), 2 (light brown), 3 (pink), 4 (dark red); Taste: 1 (not sour), 2 (slightly sour), 3 (sour), 4 (very sour); Aroma: 1 (not smoky aroma), 2 (slightly smoky aroma), 3 (smoky aroma), 4 (very smoky aroma); Texture: 1 (not chewy), 2 (somewhat chewy), 3 (chewy), 4 (very chewy)

Table 5 shows the organoleptic characteristics of fermented sausages without and with the addition of jack bean flour. The addition of jack bean flour significantly impacted both the hedonic testing and hedonic quality evaluation of fermented sausages. It altered color, taste, aroma, and texture aspects in both tests. Fermented sausages with jack bean flour had a light brown color, slightly acidic taste, and non-chewy texture, but were less preferred overall. This could be due to the bitter aftertaste from HCN in jack bean flour [29,48] and its hygroscopic properties [28], leading to drier and less chewy sausages. However, they exhibited a stronger smoky aroma, likely enhanced by the Maillard reaction during jack bean flour production [49]. According to Daun [50], the brown color is caused by carbonyl compounds such as acetol, glycolaldehyde, and methylglyoxal in the smoke. These changes are influenced by factors such as smoking process and sausage positioning during smoking [51]. Overall, panelists preferred sausages without jack bean flour for their acidic taste, somewhat chewy texture, and favorable aroma.

Conclusion

Adding jack bean flour to premium IPB fermented lamb sausages enhanced various attributes such as pH, springiness, chewiness, crude protein, and carbohydrate content, total unsaturated fatty acids, and essential amino acids. It also lowered total acid value, water activity, gumminess, water content, and the counts of *E. coli* and *S. aureus* bacteria. These sausages meet the Indonesian National Standard for Meat Sausages 3820–2015 regarding water, fat, and crude protein content. The sensory aspects such as color in fermented sausages with the addition of jack bean flour were preferred by the panelists. Fermented sausages with the addition of jack bean flour can be considered a superior choice in terms of nutritional quality and food safety compared to fermented sausages without the addition of jack bean flour.

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CORIANDER AS A NATURAL ANTIMICROBIAL FOR MEAT PRODUCTS: A ONE HEALTH PERSPECTIVE REVIEW

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Keywords: *plant-based preservation, essential oils, foodborne pathogens, food safety, phytochemicals*

Abstract
The demand for safe, high-quality meat products drives the need for effective antimicrobial solutions to combat bacterial contamination, a significant health and economic concern. Synthetic preservatives face increasing scrutiny, leading to interest in natural alternatives such as coriander (Coriandrum sativum L.). Known for its culinary and medicinal uses, coriander essential oils, particularly linalool and pinene, exhibit strong antimicrobial properties against a wide range of pathogens. This review examines the phytochemical composition and antimicrobial mechanisms of coriander, and its practical applications in meat preservation through a One Health perspective, which addresses the interconnectedness of human, animal, and environmental health. Coriander offers unique benefits such as a milder flavor and cost-effectiveness. Despite challenges, including variability in antimicrobial efficacy and sensory impacts, its safety profile and regulatory status support its use. Future research should optimize extraction methods, explore synergies with other preservatives, and evaluate long-term safety and efficacy. Coriander is a viable natural solution for improving food safety and quality in the meat industry, aligning with One Health objectives by promoting sustainable practices and reducing health risks across the food production continuum.

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Introduction

The quest for safe and high-quality meat products has never been more crucial, especially in an era where the global food supply chain faces unprecedented challenges. Bacterial contamination in meat products is a persistent and alarming issue, with pathogens such as *Salmonella* species, *Escherichia coli*, and *Listeria monocytogenes* frequently implicated in severe foodborne illnesses [1]. These microorganisms can infiltrate meat at various points of the production process, from slaughter and processing to packaging and storage, driven by lapses in hygiene practices, mishandling, and inadequate refrigeration [2]. The consequences are profound with significant health risks to consumers and substantial economic losses to the meat industry [3].

In light of these challenges, the search for effective antimicrobial agents has taken on heightened urgency. Traditional synthetic preservatives, while effective, are increasingly scrutinized for their potential health risks and environmental impact [4]. This has spurred a growing interest in natural antimicrobial agents, which offer a safer and more sustainable alternative. Natural antimicrobials, derived from plants, herbs, and other biological sources, are valued for their ability to inhibit microbial growth without adverse effects on human health or the environment [5].

Among the array of natural antimicrobials, coriander (*Coriandrum sativum L.*) stands out as a particularly promising candidate. Known for its culinary versatility and medicinal properties, coriander has been used for centuries in various cultures [6]. Recent scientific investigations have begun to uncover its potent antimicrobial properties, revealing its potential as a natural preservative in meat products [7,8,9]. Coriander essential oils and bioactive compounds exhibit a broad spectrum of activity against a range of pathogenic bacteria, positioning it as a viable solution to enhance food safety and quality [10].

Furthermore, from a One Health perspective, the use of natural antimicrobials such as coriander not only addresses food safety but also promotes environmental sustainability [11]. By reducing the reliance on synthetic preservatives and embracing natural alternatives, we can minimize the ecological footprint of meat production, decreasing chemical residues and pollution. This holistic approach ensures that our efforts to safeguard public health are in harmony with the need to protect and preserve our environment for future generations.

This review delves into the multifaceted role of coriander, exploring its phytochemical composition, antimicrobial mechanisms, and practical applications in meat preservation. By examining the evidence and advancements

in this field, we aim to highlight how coriander can contribute to mitigating bacterial contamination in meat products, thereby enhancing public health and consumer confidence. Additionally, we will explore how the use of coriander aligns with the One Health approach by addressing the interconnected impacts on human health, animal health, and environmental sustainability.

Objects and methods

The methodology for this review involved a systematic and interdisciplinary literature search. The process was designed to encompass various dimensions of antimicrobial efficacy, meat safety, and holistic health impacts, integrating insights from fields such as microbiology, food science, and public health.

The advanced search methodology used to conduct the study consisted of two stages. The initial stage of the literature search involved identifying and gathering relevant studies to address the objectives of the review. This was achieved through an extensive search using predefined keywords and phrases related to antimicrobial properties of coriander and meat contamination. The key terms included "plant-based preservation, essential oils, foodborne pathogens, food safety, and phytochemicals". The search was conducted in multiple databases to ensure a broad and inclusive collection of relevant literature such as PubMed, Scopus, Google Scholar, ScienceDirect, and Web of Science. These databases were selected for their comprehensive coverage of scientific articles, reviews, and book chapters related to microbiology, food safety, and public health. The second stage involved a meticulous selection process. It began with screening the titles and abstracts of the collected studies to identify those that were most pertinent to the focus of the review. Publications were evaluated based on their relevance to antimicrobial effects of coriander, its application in meat safety, and the integration of a One Health perspective.

Inclusion criteria were as follows:

1. Peer-reviewed articles and reviews published between 2005 and 2024.
2. Studies that investigated the antimicrobial properties of coriander or its extracts in meat products.
3. Research articles addressing the application of natural antimicrobials from a One Health perspective.
4. Papers with clear experimental data or case studies related to effectiveness of coriander in meat preservation.

Exclusion criteria were as follows:

1. Articles not related to coriander or natural antimicrobials in the context of meat preservation.
2. Publications that lack experimental data or are purely theoretical without practical application.

Data sources and geographic information:

The data sources included primary research studies, review articles, and meta-analyses. Geographic information was considered to include a diverse range of studies from different regions to ensure comprehensive coverage. The literature reviewed included research from North America, Europe, Asia, and Africa, reflecting global perspectives on the use of coriander as an antimicrobial agent in meat products.

Research subjects and analysis techniques:

The research subjects focused on various types of meat products including beef, poultry, and fish. Studies were analyzed for their methodologies in assessing the antimicrobial efficacy of coriander, such as the use of *in vitro* tests, sensory evaluations, and shelf-life studies. Data were synthesized to highlight common findings and discrepancies in effectiveness of coriander across different meat types and conditions.

Analysis techniques involved qualitative synthesis and comparative analysis of the studies reviewed. Key factors assessed included the concentration of coriander extracts used, methods of application (e. g., direct application, infusion, etc.), and effectiveness against specific microbial contaminants. Statistical analysis techniques reported in the studies were also evaluated to determine the robustness of the findings.

Phytochemical composition of coriander

Coriander is renowned not only for its culinary applications but also for its rich phytochemical profile, which underpins its potential as a natural antimicrobial agent [12]. The primary active compounds in coriander are essential oils and various phytochemicals, each contributing to its biological activities [13]. The essential oils of coriander, predominantly composed of linalool (approximately 60–70%) and pinene, are of significant interest due to their potent antimicrobial properties [14]. Linalool, a monoterpene alcohol, is known for its ability to disrupt microbial cell membranes, thereby exhibiting broad-spectrum antibacterial and antifungal activities [15]. Additionally, the essential oil contains other compounds such as camphor, borneol, and geranyl acetate, which collectively enhance its antimicrobial efficacy [16]. In terms of phytochemical composition, coriander seeds also contain various bioactive compounds including flavonoids, phenolic acids, and terpenoids [17]. These compounds contribute to the antioxidant, anti-inflammatory, and antimicrobial properties of coriander [18]. For instance, the presence of quercetin and kaempferol in coriander seeds has been associated with its ability to scavenge free radicals and inhibit the growth of pathogenic microorganisms [19].

The extraction of essential oils and phytochemicals from coriander is achieved through several methods, each affecting the yield and composition of the extract [20]. Steam distillation is the most common technique used to obtain coriander essential oil [21]. This method involves passing steam through coriander seeds, causing the essential oil to evaporate and subsequently condense into a liquid form [22]. Alternatively, solvent extraction and cold pressing are employed to obtain extracts and oils, respectively [23]. Solvent extraction uses organic solvents, such as ethanol or hexane, to dissolve the essential oils, which

are then separated from the solvent [24]. Cold pressing involves mechanically pressing the seeds to extract the oil without the use of heat, preserving its sensitive compounds [25]. Overall, the diverse array of active compounds in coriander, coupled with the various extraction methods employed, underscores its potential as a valuable natural antimicrobial agent [26]. This phytochemical richness makes coriander a compelling prospect for further exploration and application in food safety and preservation.

Antimicrobial properties of coriander

Coriander exhibits significant antimicrobial properties, making it a valuable option for enhancing food safety. The essential oils and phytochemicals derived from coriander demonstrate various mechanisms of action against bacterial pathogens, contributing to their efficacy as natural antimicrobial agents [27]. One of the primary mechanisms through which coriander exerts its antimicrobial effects is the disruption of bacterial cell membranes [28]. Linalool, the main component of coriander essential oil, interacts with the lipid bilayer of bacterial membranes, causing increased permeability and leading to cell leakage and death [29]. This mechanism is effective against a broad spectrum of bacteria, including both Gram-positive and Gram-negative strains [30]. Additionally, other compounds in coriander essential oil, such as pinene and camphor, also contribute to membrane disruption and subsequent microbial inhibition [31].

Coriander has been extensively studied for its antimicrobial properties through various *in vitro* experiments, demonstrating its effectiveness against a wide range of bacterial pathogens. Essential oil and extracts of coriander have shown substantial antimicrobial activity, which has been verified through different methodologies. Talebi et al. [7] conducted a study to assess the antimicrobial activity of coriander essential oil using agar diffusion technique. Their results revealed significant bacterial growth inhibitory properties against both Gram-positive (*S. aureus)* and Gram-negative (*P. aeruginosa* and *E. coli*) bacteria at 100% concentration of essential oil. Teshale et al. [32] performed a comprehensive *in vitro* evaluation using the disc diffusion method, finding that coriander oil (15 µL/disc) demonstrated the antibacterial activity against *E. coli*, *P. aeruginosa*, and *Salmonella* typhi (*S.* typhi), with inhibition zone diameters of 25 mm, 10 mm, and 18 mm, respectively. At this concentration, coriander oil exhibited bactericidal effects against *S.* Typhi and bacteriostatic effects against *E. coli*. In addition, Zare-Shehneh et al. [33] utilized a MIC assay to show that coriander essential oil exhibited antimicrobial activity against Gram-negative bacteria, with minimum inhibitory concentrations (MIC) of 71.55 µg/mL for *K. pneumoniae* and 86.4 µg/mL for *P. aeruginosa*, as well as against Gram-positive bacteria, with a MIC of 35.2 µg/mL for *S. aureus*. This is consistent with findings by Nanasombat and Lohasupthawee [34], who demonstrated the inhibitory effect of coriander EO against 25 bacterial strains including 20 serotypes of *Salmonella* and five other enterobacteria species: *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Serratia marcescens*. The results showed a minimum inhibitory concentration (MIC) of 4.2 µL/mL for most bacterial strains. However, *Salmonella enterica* serotype Rissen was resistant to *Coriandrum sativum* essential oil, with a MIC greater than 62.5 µL/mL. In another investigation conducted by Elkady et al. [9], using 1%–2% coriander oil reduced the *E. coli* count in camel meat by 24% and 65%, respectively, without affecting the sensory characteristics. These *in vitro* studies collectively underscore the robust antimicrobial properties of coriander. They reveal its potential as an effective natural preservative capable of combating a diverse spectrum of bacterial pathogens, supporting its application in food safety and preservation.

Application of coriander in meat products

Coriander can be incorporated into meat products through various methods to harness its antimicrobial properties. One common approach is the direct addition of coriander essential oil or ground coriander seeds during the preparation and processing of meat products. Essential oil can be emulsified and evenly distributed in meat matrices, whereas ground seeds can be mixed into meat batters or coatings [35]. Another innovative method involves using coriander extracts as a marinade for meats, allowing the bioactive compounds to permeate meat and exert their antimicrobial effects [36]. Additionally, coriander can be integrated into packaging materials or edible films that encase meat products, providing a sustained release of antimicrobial agents during storage [37].

Vacuum packaging or modified atmosphere packaging (MAP) combined with coriander essential oil or extracts can significantly extend the shelf life of meat by inhibiting the growth of spoilage microorganisms and pathogens [38]. Edible coatings enriched with coriander compounds can be applied to the surface of meat products, creating a barrier that slows down microbial growth and oxidation processes [39]. Moreover, coriander extracts can be used in combination with other natural preservatives such as lactic acid bacteria or plant-derived antimicrobials to create synergistic effects, enhancing the overall preservation efficacy [40].

The effectiveness of coriander as a natural antimicrobial agent in real-world applications has been demonstrated in several studies. For instance, research has shown that coriander essential oil exhibits potent antibacterial activity against some Gram-positive and Gram-negative bacteria (*Salmonella* Typhimurium, *Listeria monocytogenes*, *Staphylococcus aureus*, *Serratia grimesii*, *Enterobacter agglomerans*, *Yersinia enterocolitica*, and *Bacillus cereus*) [41]. Also, the antimicrobial activity of coriander against potential spoilage bacteria, including *K. pneumoniae*, *Bacillus megaterium*, *P. aeruginosa*, *S. aureus*, *E. coli*, *Enterobacter cloacae,* and *Enterococcus faecalis*, has been documented [42]. In practical applications, meat products treated with coriander extracts or essential oils have exhibited reduced microbial counts and

extended shelf life compared to untreated controls [9,43,44]. Consumer acceptability studies also indicate that coriander-treated meat products maintain desirable sensory qualities, with no significant adverse effects on taste or texture [9,45,46]. These findings underscore the potential of coriander as an effective and natural solution for enhancing the microbial safety and quality of meat products. By integrating coriander into meat products through these methods and preservation techniques, the meat industry can leverage its natural antimicrobial properties to mitigate bacterial contamination, improve food safety, and potentially reduce the reliance on synthetic preservatives.

Comparative analysis with other natural antimicrobials

Compared to other natural preservatives such as garlic, thyme, rosemary, and oregano, coriander offers unique benefits and similar effectiveness [47]. Coriander essential oil demonstrates broad-spectrum antimicrobial activity against foodborne pathogens including *Salmonella* species, *Listeria monocytogenes*, and *Escherichia coli*, comparable to garlic and thyme [48]. Its milder aroma and flavor make it preferable for preserving the sensory qualities of meat products [49]. Active compounds of coriander, such as linalool and geraniol, are effective at low concentrations, making it a cost-effective option [13]. Combining coriander with other antimicrobials, for example oregano or thyme essential oils, enhances its effectiveness due to synergistic interactions [50]. Additionally, pairing coriander with organic acids such as citric or lactic acid improves antimicrobial efficacy and meat quality during extended storage [51]. This strategic use of coriander and other natural preservatives offers a promising approach to enhancing microbial safety and extending shelf life while maintaining desirable sensory attributes.

Impact on sensory and nutritional qualities of meat

Incorporating coriander into meat products can significantly influence their sensory attributes, including taste, aroma, and texture. Coriander essential oil and ground seeds impart a distinct flavor profile characterized by citrusy, nutty, and slightly spicy notes. This can enhance the overall palatability of meat, making it more appealing to consumers [52]. Studies have shown that moderate levels of coriander addition are generally well-received, as the mild and pleasant aroma does not overpower the natural taste of meat [53,54]. Moreover, volatile compounds of coriander can help mask any undesirable odors that might develop during storage [55]. However, excessive use of coriander might lead to an overpowering flavor that could be undesirable for some consumers. In terms of texture, coriander does not significantly alter meat physical properties, ensuring that a product remains tender and juicy [56]. In addition, the use of coriander in marinades and coatings can also contribute to a more appealing and consistent texture by promoting even moisture distribution [51]. Furthermore, adding coriander to meat products can enhance their nutritional profile. Coriander seeds and leaves are rich in essential nutrients, including vitamins A, C, and K, as well as minerals such as potassium, calcium, and magnesium [57] These nutrients can fortify meat, contributing to a healthier final product. Additionally, coriander contains dietary fiber and antioxidants, which can offer health benefits such as improved digestion and reduced oxidative stress [58]. The antimicrobial compounds in coriander, primarily linalool and geraniol, not only help in preservation but also have potential health benefits, including anti-inflammatory and anti-carcinogenic properties [59]. Therefore, the inclusion of coriander can boost the functional value of meat products, aligning with the growing consumer demand for healthier food options. By incorporating coriander, meat producers can create products that are not only safer and longer-lasting but also offer enhanced sensory and nutritional qualities. This makes coriander a multifaceted ingredient that can meet both preservation and consumer satisfaction goals in the meat industry.

Safety and regulatory aspects

Toxicological studies and safety evaluations of coriander are essential to ensure its safe use as an antimicrobial agent in meat products. Various studies have demonstrated that coriander and its essential oils are generally safe for human consumption at the concentrations typically used in food preservation [55]. Acute and chronic toxicity studies on animals have shown that coriander essential oil has a high safety margin, with no significant adverse effects observed at doses considerably higher than those used in food applications [41]. Additionally, the main bioactive compounds of coriander, such as linalool and geraniol, have been evaluated for genotoxicity and carcinogenicity, with results indicating no mutagenic or carcinogenic potential [60,61]. These findings support the conclusion that coriander is a safe natural additive for enhancing the microbial safety of meat products.

The regulatory status of coriander as a food additive varies across different regions but is generally favorable due to its long history of safe use in culinary applications [62]. In the United States, coriander and its essential oil are classified as Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA), meaning they can be safely used in foods within certain limits without requiring pre-market approval [63]. Similarly, the European Food Safety Authority (EFSA) has evaluated coriander and deemed it safe for use as a flavoring agent in various food products [64]. Despite these approvals, it is important for food manufacturers to adhere to regulatory requirements and guidelines when incorporating coriander into meat products. This includes complying with labeling regulations, ensuring that the levels of coriander used do not exceed permissible limits, and conducting regular safety assessments to monitor any potential adverse effects. By

following these regulatory frameworks, the meat industry can confidently utilize coriander as a natural antimicrobial agent, enhancing food safety while maintaining compliance with food safety standards.

Integrating One Health principles in the use of coriander for controlling bacterial contamination in meat products

The One Health framework advocates for a comprehensive approach to managing health risks by considering the interdependencies between human, animal, and environmental health [65]. Coriander as a natural antimicrobial in meat products can significantly enhance food safety, directly impacting public health. Meat products contaminated with pathogenic bacteria pose a serious risk to human health, often leading to foodborne illnesses that can strain healthcare systems [66]. By utilizing antimicrobial properties of coriander, we can reduce the prevalence of bacterial contamination in meat, thereby lowering the incidence of foodborne diseases. This approach not only protects consumers but also aligns with One Health objective of mitigating risks at the human-animal-environment interface. Antimicrobial resistance (AMR) is a critical concern in both human and animal health, driven by the overuse and misuse of antibiotics in livestock production [67]. One Health emphasizes the importance of addressing AMR through integrated strategies. The use of coriander as a natural antimicrobial in meat products presents a sustainable alternative to conventional antibiotics, thereby reducing the selective pressure that drives AMR [68]. By incorporating coriander into meat processing, we can help decrease the reliance on synthetic antimicrobials, ultimately contributing to the preservation of antibiotic efficacy. This aligns with One Health goal of combating AMR by fostering practices that minimize the spread of resistant bacteria across the human-animal-environment continuum.

The One Health approach also highlights the importance of sustainable practices in maintaining ecosystem health [69]. Coriander, as a natural antimicrobial, supports sustainability in meat production by reducing the environmental burden associated with synthetic chemicals and antibiotics. The cultivation of coriander itself is associated with minimal environmental impact compared to synthetic alternatives [70]. Furthermore, integrating coriander into meat processing aligns with the principles of sustainable agriculture, which aim to enhance biodiversity, reduce chemical runoff, and promote soil health [71]. By utilizing coriander, which can be grown with relatively low inputs and minimal impact on the surrounding ecosystem, we contribute to more sustainable and environmentally friendly food production systems. This practice not only supports the One Health objective of protecting environmental health but also ensures that food production methods remain resilient and adaptable to future challenges.

One Health emphasizes the need for collaborative efforts across different sectors to address complex health issues effectively [72]. The incorporation of coriander as a natural antimicrobial in meat processing exemplifies how interdisciplinary approaches can lead to significant improvements in food safety. Collaboration between agricultural scientists, food technologists, public health experts, and environmentalists is crucial for maximizing the benefits of coriander in controlling bacterial contamination. Such interdisciplinary cooperation can facilitate the development of best practices for coriander application, ensuring that its antimicrobial properties are utilized effectively while also considering its impact on the environment and overall food safety. By fostering cross-sector partnerships, we can advance One Health goals, create more robust food safety systems, and promote a holistic approach to managing health risks at the human-animal-environment interface.

Integrating coriander into meat processing also provides an opportunity to enhance public health education and policy within the One Health framework. Educating consumers, producers, and policymakers about the benefits of natural antimicrobials like coriander can drive more informed decisions regarding food safety practices and regulatory policies. Promoting the use of coriander as a viable alternative to synthetic antimicrobials can support policy initiatives aimed at reducing antibiotic use in agriculture and improving overall food safety standards. Additionally, raising awareness about the One Health implications of natural antimicrobial use can help align public health strategies with sustainable and environmentally conscious practices, further advancing the objectives of the One Health approach.

Challenges and limitations

While coriander shows promise as a natural antimicrobial agent for meat products, several potential drawbacks and limitations must be considered. One of the primary limitations is the variability in the antimicrobial efficacy of coriander essential oil and extracts, which can be influenced by factors such as the geographical origin of the coriander, the method of extraction, and storage conditions [52]. This variability can lead to inconsistent results in microbial inhibition. Additionally, while coriander has a relatively mild flavor, its strong, distinct aroma might not be universally accepted by all consumers, potentially affecting the sensory qualities of meat products [55]. Furthermore, the effectiveness of coriander as an antimicrobial agent is often concentration-dependent, and higher concentrations required for significant microbial inhibition may not be feasible due to cost constraints and potential sensory impacts [73]. Implementing coriander as a natural antimicrobial agent in large-scale meat processing faces several challenges. Standardizing coriander extracts or essential oils for consistent antimicrobial activity requires rigorous quality control, which can be resource-intensive. Integrating coriander into existing workflows may necessitate modifications to equipment and processes, increasing operational costs [74]. Ensuring the stability and efficacy of antimicrobial compounds of coriander throughout the

meat product shelf life is another concern, as factors such as temperature, humidity, and light can degrade the bioactive compounds [75]. Additionally, while generally safe, coriander can cause allergic reactions or sensitivities in some individuals, necessitating clear labeling and consumer education [76]. Overall, while coriander has significant potential as a natural antimicrobial agent for meat products, these challenges must be carefully managed for effective and safe large-scale application.

Future prospects and research directions

The use of natural antimicrobials in food preservation is a rapidly growing field, driven by increasing consumer demand for clean-label products and the need to reduce reliance on synthetic preservatives. Emerging trends in this area include the exploration of plant-based extracts, essential oils, and bioactive compounds with antimicrobial properties. Researchers are focusing on identifying new sources of natural antimicrobials and understanding their mechanisms of action. Additionally, there is a trend towards developing multifunctional natural preservatives that not only inhibit microbial growth but also enhance the sensory and nutritional qualities of food products. Technological advancements, such as nanoencapsulation and microencapsulation, are being applied to natural antimicrobials to improve their stability, controlled release, and efficacy. These technologies protect the bioactive compounds from degradation and ensure their sustained antimicrobial activity over the product shelf life.

Furthermore, the combination of natural antimicrobials with other preservation methods, such as high-pressure processing and pulsed electric fields, is being explored to enhance food safety and extend shelf life. Several areas warrant further research and development to optimize the use of coriander as a natural antimicrobial in meat products. One key area is the standardization of coriander extracts and essential oils. This involves developing methods to ensure consistent quality and antimicrobial efficacy across different batches. Research should focus on identifying the optimal extraction techniques and conditions that maximize the yield and activity of the bioactive compounds in coriander.

Another critical area is the investigation of the synergistic effects of coriander with other natural antimicrobials and preservation methods. Understanding how coriander interacts with other compounds can help in formulating more effective antimicrobial systems. Studies should explore the optimal combinations and concentrations that provide the best antimicrobial activity without compromising the sensory qualities of meat. Further research is also needed to evaluate the long-term safety and efficacy of coriander in various meat products under different storage conditions. This includes studying the stability of antimicrobial compounds of coriander and their interactions with meat components over time. Additionally, research should address the potential allergenicity and consumer acceptance of coriander-treated meat products.

Lastly, advancements in biotechnology and genetic engineering offer opportunities to enhance the antimicrobial properties of coriander. Genetic modifications could be explored to increase the production of bioactive compounds in coriander plants, potentially leading to more potent antimicrobial agents.

Conclusion

Coriander (*Coriandrum sativum L.*) is a promising natural antimicrobial for meat preservation due to its rich phytochemical composition, particularly linalool, flavonoids, and phenolic acids. These compounds effectively combat Gram-positive and Gram-negative bacteria, enhancing food safety and extending shelf life without compromising sensory qualities. Its use supports the One Health approach by promoting human health, sustainable agriculture, and environmental protection. However, challenges like antimicrobial variability, extract standardization, and consumer acceptance need addressing. Future research should focus on optimizing extraction, exploring synergies with other preservatives, and enhancing efficacy through technologies like nanoencapsulation.

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BIOTECHNOLOGICAL METHODS FOR SEPARATION OF PIGS PANCREAS GLANDS PROTEIN SUBSTANCES WITH MEMBRANE TECHNOLOGIES

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Keywords: *pancreas gland, electrophoresis, diafiltration, ultrafiltration, dialysis, trehalose, glycine, proline*

Abstract
The pancreas gland (PG) is a secondary product of livestock processing; it contains a wide range of biologically active compounds. The purpose of this article is to analyze the efficiency of technological approaches for pancreas gland extraction with the help of trehalose and a glycine-proline mixture aimed for recovery and separation of the gland's protein-peptide compounds. The extraction was conducted with 0.9% NaCl, 0.9% NaCl, with addition of 0.5 M trehalose (0.9% NaCl-0.5 M trehalose) and 0.9% NaCl with addition of 1% glycine and 0.1 M L-proline (0.9% NaCl-1% Gly-0.1M Pro), the ratio of pancreas gland to extractant was equal to 1:5. The concentration of the protein in the supernatants after their extraction was measured by the biuret reaction in a semi-automatic biochemical analyzer Biochem SA. The proteomic composition of the extracts and the native pancreas gland was assessed by one-dimensional Laemmli electrophoresis in a 12.5% polyacrylamide gel and by two-dimensional O'Farrell electrophoresis. When determining the intensity of the protein fractions, it was noted that the methodology of separation of protein-peptide mixtures extracted from the pigs pancreas gland with the extractant 0.9% NaCl-1% Gly-0.1M Pro, ensured the higher extraction of the proteins in comparison with the method of 0.9% NaCl-0.5 M trehalose. Notwithstanding the fact that application of amino acids (glycine and proline) mixture provided for a greater yield of proteins from the extract into the diafiltrate, the experiments in vitro showed that the diafiltrate obtained though trehalose featured higher activity. This may be explained by the fact that after dialysis removal of trehalose from the protein fraction with a molecular weight of less than 50 kDa, its residual quantities were still sufficient to prevent proteins aggregation and, as a consequence, the biological activity of the extracted proteins was preserved, while in the diafiltrate obtained through amino acids mixture where numerous protein aggregates were detected by 2-DE. This study allowed testing the biotechnological methodics on pig pancreatic tissues aimed to intensifying the extraction and separation of protein compounds. The results of the study are important for development of methodological approaches to obtaining the targeted substances for their further utilizing for various purposes.

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Introduction

The raw materials of animal origin, as well as secondary products of their processing, contain a wide assortment of biologically active compounds with various purposefulness and mechanisms of action. To prevent metabolic disorders related to age and diet, which are predictors of diabetes mellitus, obesity, dyslipidemia, and cardiovascular diseases [1], food products and dietary supplements are widely used [2]. A promising source of bioactive substances of animal origin is the pancreas gland (PG), which produces a huge amount of endocrine and exocrine compounds and can serve as a source of hormones, enzymes and their precursors, structural, regulatory, secretory and receptor substances [3]. In particular, the medications like insulin, glucagon, trypsin, chymotrypsin, and pancreatin are produced from the pancreas gland [4,5].

The extraction of some individual valuable protein substances from the pancreas gland is pretty challenging, so new technological approaches are currently being sought. One of the promising directions of researches is membrane technologies, which is an effective option for the separation, fractionation and purification of biologically active compounds obtained from various animal tissues [6]. Depending on the purpose, various types of filtration processes, various materials and polymers are used for producing the membranes.

Ultrafiltration is a widely used method for proteins separation, fractionation and purification. However, the issues of adsorption, aggregation of protein molecules and their denaturation are the main challenges that can be faced during the ultrafiltration of proteins in native form [7,8]. The simplest ways to prevent proteins aggregation and adsorp-

tion during their ultrafiltration are dilution followed by diafiltration to maintain a constant pH and ionic strength [9,10] of the mixture exposed to separation. The selection of membrane material, optimal pore size and the rate of penetration of the protein solution through it also affect the optimization of the ultrafiltration process [11]. In addition, sugars and polyhydric alcohols, including glucose, sucrose, trehalose, lactose, glycerol, sorbitol, mannitol, xylitol, and inositol, are also widely used to prevent protein aggregation. It has been suggested that trehalose may stabilize protein structure and may also prevent proteins from interacting with each other [12].

Also, the use of amino acids as anti-aggregating (anti-clogging) agents is in demand in the food industry and the manufacture of dietary supplements. According to the researchers, the pharmacological activity of a substance increases after it is combined with an amino acid, and its solubility in water also improves and cytotoxicity decreases [13]. Glycine, for example, stabilizes aggregated conformations of hydrophobic elastin-like polypeptides through the classical preferential depletion mechanism (traditional depletion mechanism) [14]. Glycine and proline have shown some ability to stabilize hemoglobin [15]. Experimental data demonstrate that proline inhibits protein aggregation by binding to a folding intermediate product and converting it to an enzymatically inactive, "aggregation-insensitive" state [16].

The purpose of the work was to study the efficiency of technological approaches for the separation of protein-peptide substances of pig pancreas glands extracted using extractants containing trehalose and a glycine-proline mixture.

Objects and methods

The object of the study was the pancreas glands of pigs, obtained from Pushkinsky Myasnoy Dvor LLC, Moscow region, Pushkino town. Animal raw materials were trimmed of connective tissue, frozen at minus 18 °C, then crushed and frozen down to minus 40 °C until further extraction. The crushed pancreas gland (PG) was thawed at a temperature of 4 °C and mixed with the extractant in a ratio of 1:5. Extraction was run in a laboratory dispersing unit LDU-3 MPR (Labotex, Russia) with a speed of steering at 400 rpm; extraction lasted 150 min at 4 °C.

To study the efficiency of technological approaches for the separation of protein-peptide compounds extracted from pancreas glands, a series of experiments were conducted. In total of 3 extractions were carried out with the following extractants:

1) 0.9% sodium chloride solution (Gematek LLC, Russia);
2) 0.9% sodium chloride solution (Gematek LLC, Russia) with addition of 0.5 M trehalose (Narodnaya Zdrava, Russia) (0.9% NaCl-0.5 M trehalose);
3) 0.9% sodium chloride solution (Gematek LLC, Russia) with addition of 1% (mass fraction) glycine (PanReac AppliChem, Germany), 0.1 M L- proline (Sigma-Aldrich, USA) (0.9% NaCl-1% Gly-0.1 M Pro).

Upon extraction process completion, the supernatant was separated by centrifugation for 5 minutes at a speed of 3500 rpm on a centrifuge CM-6M (ELMI, Latvia), after that it was filtered through a cotton-gauze filter to clear from suspended matter. Part of the extract was frozen at minus 40 °C in a freezer IW-401–262 Deep Freezer (Haier, China) followed by its freeze-drying on a laboratory freeze-dryer LS-1000 (Prointeh-bio, Russia). Part of it was saved for further measuring of the protein concentration in the extract, for ultrafiltration and diafiltration. In each sample, the protein concentration was measured by a biuret reaction on a semi-automatic biochemical analyzer Biochem SA (HTI, USA) with the help of the commercial Total Protein reagent (HTI, USA). The measurements were run in triplicate.

To determine the most effective approach to the separation of biologically active protein-peptide mixtures from pigs pancreatic tissues, the ultrafiltration and diafiltration of pancreatic extracts obtained using extractants 0.9% NaCl-0,5 M trehalose and 0.9% NaCl-1% Gly-0.1M Pro were conducted. Press-type ultrafiltration was run in the centrifugal ultrafilters Amicon Ultra-4 50 kDa (Millipore, Germany), the regenerated cellulose was used as the material of the membrane. To run the diafiltration, the device Vivaflow 200 (Sartorius, Germany) was used, polyether sulfone was used as the membrane material, the pass-through capacity amounted to 50 kDa. The resulting ultrafiltrates and diafiltrates were frozen at minus 40 °C in a freezer IW-401–262 Deep Freezer (Haier, China) followed by freeze drying LS-1000 (Prointeh-bio, Russia).

To purify the extracts, ultrafiltrates (UF) and diafiltrates (DF) obtained after extraction with 0.9% NaCl-0.5 M trehalose and 0.9% NaCl-1% Gly-0.1 M Pro, a dialysis method was used, in which method the gradient of concentration promoted the release of low molecular weight substances through the semi-permeable membrane. SnakeSkin Dialysis Tubing regenerated cellulose dialysis bags were used for dialysis (Thermo Fisher Scientific, USA) with a pass-through capacity of 3.5 kDa and a diameter of 16 mm. The samples after dialysis were frozen at minus 40 °C in a freezer IW-401–262 Deep Freezer (Haier, China) followed by freeze drying in the device LS-1000 (Prointech-bio, Russia).

Analysis of molecular weight distribution of the protein fractions in the freeze-dried samples of native pancreas glands, extracts before and after dialysis, UF and DF after dialysis was run by one-dimensional electrophoresis according to Laemmli [17]. For preparation of the sample of freeze-dried native pancreas gland, 2000 mcl of lysis solution was used (9 M urea (PanReac, Germany), 5% β- mercaptoethanol (PanReac, Germany), 2% Triton X-100 (Helicon, Russia), 2% ampholines pH 3–10 (Serva, Germany)). Freeze-dried samples of pancreatic extracts before and after dialysis, as well as UF- and DF-s after dialysis, were diluted in a minimum volume of distilled water, after which 100 mcl of the sample was mixed with 100 mcl of protein buffer (1 ml sodium do-

decyl sulfate (SDS, Panreac, Spain) 10%, 250 µl concentrated β- mercaptoethanol (PanReac, Germany), 625 µl Tris-HCl 0.5 M (PanReac, Germany), 1.5 g urea (PanReac, Germany), bromophenol blue (Helicon, Russia) and heated in a boiling water bath in the device Assistant 26026–1 (Karl Hecht, Germany) for 5 minutes. Next, the resulting homogenate solution was centrifuged at 14,000 rpm for 20 minutes on an Eppendorf 5427 R centrifuge (Eppendorf, Germany). To run one-dimensional electrophoresis, the "VE-10" chamber (Helicon, Russia) was used; it was filled with 12.5% polyacrylamide gel.

O'Farrell electrophoresis was performed using the Bio-Rad camera (Bio-Rad (USA)) with isoelectric focusing (IEF) in glass tubes in the first direction and SDS-PAGE in the second direction, as described by Matsumoto N. [18] with slight modifications [19]: IEF in the first direction was performed in 2.4 mm × 160 mm tubular gels until reaching 3,650 volt-hours. To run the two-dimensional electrophoresis, 100 mg of pancreatic diafiltrate after dialysis was taken and mixed with 1000 mcl of distilled water. The mixture was stirred for 10 minutes at rate of 600 rpm using a MPS-1 vortex (BioSan, Latvia). The resulting homogenate was used in isoelectric focusing. Sedimentation of the samples was also conducted in order to concentrate the proteins and purify them from the various contaminants. For this the samples of diafiltrates were mixed with 10% TCA (PanReac, Spain) in a ratio of 1 to 1 and incubated for 15 hours at a temperature of +4–6 °C. After that the centrifugation was run at rate of 10,000 rpm for 10 minutes at 4 °C on an centrifuge Eppendorf 5427 R (Eppendorf, Germany). The supernatant was poured off, then the resulting precipitate was dissolved with lysis buffer (9 M urea (PanReac, Germany), 5% β-mercaptoethanol (PanReac, Germany), 2% Triton X-100 (Helicon, Russia), 2% ampholines with pH 3–10 (Serva, Germany)). The obtained protein extracts were used for isoelectric focusing.

Marker composed of preparations –standards of molecular weights — was used as a standard solution. (Thermo Scientific, USA). Protein staining was conducted in the solution of the following composition: 10% acetic acid (Component-Reaktiv; Russia), 25% isopropanol (PanReac, Germany), 0.05% coomassie G-250 (Helicon, Russia). To remove the unbound dye, 10% acetic acid (Component-Reaktiv; Russia) was used. To increase resolution capacity, the additional staining was run with silver nitrate (PanReac, Germany) according to Blum's method [20].

In order to run the computer densitometry, wet electropherograms were used. Their full digital images were obtained via the scanner Bio-5000 Plus (Serva, Germany) in 600 ppi mode for one-dimensional electropherograms, and 300 ppi for two-dimensional electropherograms, 2D-RGB and 1D-Gray. The resulting 2D electropherogram images were analyzed using the software ImageMaster ™ 2D Platinum based on Melanie 8.0 (GE Healthcare and Genebio, Switzerland).

To study the biological properties obtained by DF during the *in vitro* experiments, pancreas gland fragments were obtained from male mice (n = 2) of the line C57Bl/6J (8–9 weeks). Animals were euthanized in a euthanasia chamber (VetTech, UK) filled with carbon dioxide, then the abdominal cavity was opened in sterile conditions, the pancreas gland was taken out, placed in a Petri dish and poured with a cold solution (4 °C) of phosphate-buffered saline (Servicebio, China). Pancreatic tissues were washed from erythrocytes with phosphate-buffered saline, then thoroughly chopped with scissors and enzymatic disaggregation was run in a 0.1% solution of type I collagenase (Gibco, USA) at 37 °C, 20 min with constant gentle stirring (250 rpm) on a multifunctional device ImmunoChem-2200 with a function of thermal shaker (HTI, USA). The resulting pancreatic cells were filtered through a sterile nylon filter with a pore diameter of 200–250 µm, then centrifuged at 150 g, 3 minutes (Eppendorf, Germany). The supernatant was removed, and the precipitated cells were flushed 3 times with phosphate-buffered saline. After 3 flushes with phosphate-buffered saline, the isolated cells were incubated in Petri dishes (Thermo Scientific, USA) in DMEM/F12 culture medium (Servicebio, China) with the addition of L-glutamine (PanEco, Russia), 10% fetal bovine serum (HyClone, USA), 1% penicillin-streptomycin (PanReac Applichem, Germany). Petri dishes were put in a CO2-incubator BC — J 160 (Boxun, China), at 37 °C, with 5% CO_2.

After 72 hours, the culture medium was replaced with DMEM/F12 that contained the studied DF samples 0.9% NaCl-0.5 M trehalose and 0.9% NaCl-1% Gly-0.1 M Pro at the concentrations of 100 ng /ml, incubated at 37 °C, with 5% CO_2 for 6 days long. As a control reference, a cell culture was used which was cultivated in a nutrient medium without the addition of the studied samples.

On day 9, cells were removed with 0.05% trypsin-EDTA (Sigma-Aldrich, Germany). To record the number and viability of cells, an automatic counter LUNA-FL (Logos Biosystems, South Korea) was used. A 2 mcl cell suspension was mixed with 18 mcl of acridine orange/ propidium iodide dye solution, and 10 mcl of this solution was transferred to the chamber of a PhotonSlide counting plate (Logos Biosystems, Korea). During the measurement process, the following data were obtained: the total number of cells in 1 ml, the number of unstained (living) cells, the share of viable cells, histogram of cell size distribution within the selected range and a photograph of the plate contents.

The morphology of mice pancreatic cells was visually assessed using an inverted phase-contrast biological microscope MIB-R, a digital camera MC-5 and MS-View software (LOMO, Russia).

Statistical processing was conducted using the Statistica 10.0 software package. The results were presented as mean values and standard deviations (Mean ± SD). Statistical significance was calculated using nonparametric Mann–Whitney U tests. A probability of 0.05 was selected as the significant level.

Results and discussion

The results of determining the protein concentration in pancreatic extracts obtained using various extractants are presented in Table 1.

Table 1. Results of determining protein concentration in extracts using various anti-aggregating agents

Extragent	0.9% NaCl,	0.9% NaCl-0.5 M trehalose	0.9% NaCl-1% Gly-0,1 M Pro
Protein concentration, g/l	24.80 ± 0.89^{a}	$33.33 \pm 0.98^{b, c}$	$28.79 \pm 0.13^{b, d}$

$^{a-b, c-d}$ — statistically significant difference (Mann-Whitney *U*, $p < 0.05$).

It was shown that the addition of 0.5 M trehalose, 1% Gly-0.1MPro to 0.9% NaCl contributed to the yield of protein substances into the extractant — this protein content exceeded the protein content in the extract obtained with 0.9% NaCl by 34.4% ($p < 0.05$) and 16.1% ($p < 0.05$), respectively.

Freeze-dried samples obtained using various extractants are presented below in the Figure 1, which clearly demonstrates not only the color variation during the processes of extraction and purification of protein substances, but also the white color of DF after dialysis in case of using 0.9% NaCl-0.5 M trehalose as an extractant, while DF after dialysis in case of using 0.9% NaCl-1% Gly-0.1 MPro as an extractant featured yellowish tint which indicates a higher presence of protein substances.

The results of the analysis of the protein fractions distribution among the freeze-dried samples of native pancreas glands, the extracts before and after dialysis, UF and DF after dialysis, obtained with the various extractants, are presented below in one-dimensional (Figure 2) and two-dimensional (Figures 3 and 4) electropherograms.

The track 3 in the Figure 2A featured a solid colored band and the absence of clearly outlined protein fractions, which proved the destruction of proteins during dialysis of the pancreas gland extract with 0.9% NaCl-0.5 M trehalose, which phenomenon was not observed when 0.9% NaCl-1% Gly-0,1M Pro was used as an extractant. Thus, on the track 4B, the well-expressed bands of protein frac-

Figure 1. Freeze-dried samples

[I — pancreas gland (PG), II — pancreas gland extract with 0.9% NaCl-0.5 M trehalose before dialysis, III — pancreas gland diafiltrate with 0.9% NaCl-0.5 M trehalose after dialysis, IV — pancreas gland extract with 0.9% NaCl-1% Gly-0.1M Pro before dialysis, V — pancreas gland extract with 0.9% NaCl-1% Gly-0.1M Pro after dialysis, VI — pancreas gland diafiltrate with 0.9% NaCl-1% Gly-0.1M Pro after dialysis]

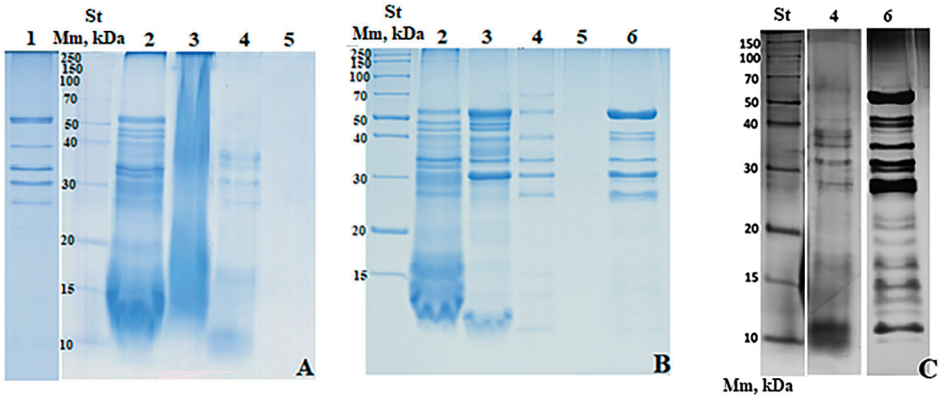

Figure 2. One-dimensional electropherograms of the samples prepared using 0.9% NaCl-0.5 M trehalose (A) and 0.9% NaCl-1% Gly-0.1 M Pro (B); fragments of DF electropherograms stained with silver nitrate (C).

Legend keys: St — molecular weight standard, kDa, 1 — pancreas gland extract (PG) with 0.9% NaCl, 2A — native pancreas gland, 3A — pancreas gland extract after dialysis, 4A — DF after dialysis, 5A — UF after dialysis, 2B — native pancreas gland, 3 B — pancreas gland extract before dialysis, 4 B — pancreas gland extract after dialysis, 5 B — UF after dialysis, 6B — DF after dialysis, 4C — DF using 0.9% NaCl-0.5 M trehalose after dialysis, stained with silver nitrate, 6C — DF using 0.9% NaCl-1% Gly-0.1M Pro after dialysis, stained with silver nitrate

tions were clearly visible, however, when compared with the track 3B, it was clear that some protein fractions were lacking, which also proved the partial destruction of proteins after dialysis. The inefficiency of press-type ultrafiltration with subsequent dialysis was determined, since there were no protein fractions on the tracks 5A and 5B. When comparing tracks 4A and 6B, it became obvious that the methodology for separating protein-peptide mixtures obtained from pancreas gland with the help of 0.9% NaCl-1% Gly-0,1M Pro as an extractant had higher efficiency than in case of using 0.9% NaCl-0.5 M trehalose. The mechanism of trehalose stabilization of proteins may partially depend on the environment as well as the type of molecule being stabilized. The mobility of biomolecules may decrease upon water binding with trehalose or vitrification, which can sometimes result to minor protein denaturation [21,22]. In its turn, proline promotes the solubility of sparingly soluble proteins and also behaves as a chaperone during protein folding [23]. In track 6C a diversity of protein fractions was observed within the range from 50 to 10 kDa, with the most expressed fractions within the band of 50, 40, 39, 35, 31, 30, 27 and 11 kDa (Figure 2 C). It can be assumed that these protein bands may correspond to pancreatic triacylglycerol lipase (50.0 kDa) [24], protein associated with pancreatic lipase (53.2 kDa) [25], phospholipase 2A (39–40 kDa) [26], elastase (27–28 kDa) [27], chymotrypsin-like elastase (28.8 kDa) [28], trypsin (24.4 kDa) [29], secretin (14.6 kDa) [30], gastrin (11.5 kDa) [31], insulin (11.6 kDa) [32] and colipase (12.1 kDa) [33]. The presented protein fractions are mainly proteolytic enzymes that implement the specific functions. Lipases are responsible for the hydrolysis of ester bonds in triacylglycerols, leading to the formation of glycerol, free fatty acids, diacylglycerols and monoacylglycerols [34]. Phospholipase A2 belongs to the phospholipid-hydrolyzing enzymes family and participates in the metabolism of phospholipids in the cell membranes, including the synthesis of prostaglandins, into the transmission of cell signals and in the serum lipoproteins metabolism [35]. Trypsin is the serine endopeptidase, which catalyzes the peptide bonds hydrolysis at the carboxyl terminus (C-terminus) of the amino acids lysine and arginine, thus releasing polypeptides. Elastase is responsible for the hydrolysis of peptide bonds on the C-terminal side of the amino acids valine, alanine and glycine with releasing of the polypeptides [36]. Secretin is a key gastrointestinal hormone involved in the regulation of pH of duodenal content [37]. Gastrin stimulates the gastric mucosa to produce hydrochloric acid and the pancreas gland to produce digestive enzymes, also stimulates the contraction of smooth muscles, enhances blood circulation and water secretion in the stomach and intestines [38]. Insulin reduces the concentration of glucose in the blood, increases permeability of cells to monosaccharides, amino acids and fatty acids, it accelerates glycolysis, the pentose phosphate cycle and glycogen synthesis in the liver [39]. Colipase is a cofactor of pancreatic lipase, which allows the lipase to get fixated at the interface of lipid and water [40].

For the convenience of two-dimensional electropherograms analyzing, the groups of proteins on them located in the same ranges of molecular weights (MW) and isoelectric points (pI), were divided into the blocks (Figures 3 and 4, Tables 2 and 3). Comparing two-dimensional electropherograms of A, B and C in Figure 3 using 0.9% NaCl-0.5 M trehalose as an extractant, it is necessary to note that the electropherogram of the extract (A) was characterized by the largest quantity of protein fractions; and the electropherogram of DF without sedimentation (C) showed the greater diversity of protein fractions in comparison with the DF electropherogram with sedimentation (B), thus proving a greater efficiency of the sample preparation without sedimentation. Within the area of molecular weights (MW) from 30 to 50 kDa and isoelectric points from 6.3 to 7.3 (block 1), 9 protein fractions were found that were present in all studied samples. However, a slight shift was observed in their molecular weights and isoelectric points (pI) towards a lesser extent when comparing the different methods of the sample preparation, which differed significantly from the extract. Thus, the isoelectric points of the proteins No. 1–9 shifted to the alkaline area in both DFs in reference to the extract, which is especially noticeable for the proteins No. 1–4. The molecular weights of the protein fractions were slightly shifted and on average were within the range of 35–38 kDa. Molecular weights and pI can shift into one direction or another due to post-translational modifications (phosphorylation, glycosylation, etc.), with the cleavage of signal sequences or the other sequences, with the formation of protein complexes, various protein isoforms, as well as due to nonspecific proteolytic cleavage and the degradation of the protein [41,42].

The fraction No. 6was the most intensely expressed in 1 block of proteins, and in the DF electropherogram without sedimentation (C) the volume of this protein fraction accounted for 25,627,460 c. u., which was 1.3 times greater than in the electropherogram A, and also 7 times greater than in electropherogram B. The intensity of the remaining protein spots was also higher in the DF electropherogram without sedimentation (C), compared to the electropherograms of the extract (A) and DF with sedimentation (B).

Within this range of molecular weights and pI, the following proteins can be found: phospholipase [26], annexin A1 [43,44], annexin A4 [45], annexin A2 [46], cathepsin B [47], cathepsin D [48], procathepsin L [49] and procathepsin H [50,51]. Presumably, the most expressed protein in block 1, which is marked by number 6, may be annexin A2 (38.8 kDa, pI 6.92) [52].

Block 2 in the electropherograms of all samples featured the availability of groups of the proteins with molecular weights from 26 to 31 kDa and pI within the range of 6.8–7.5. There were 5 protein spots found on the electropherograms A, B and C (protein fractions No. 10–14), and protein No. 13, with a MW ~of 30 kDa and pI ~7.35, was most expressed on the DF electropherogram without

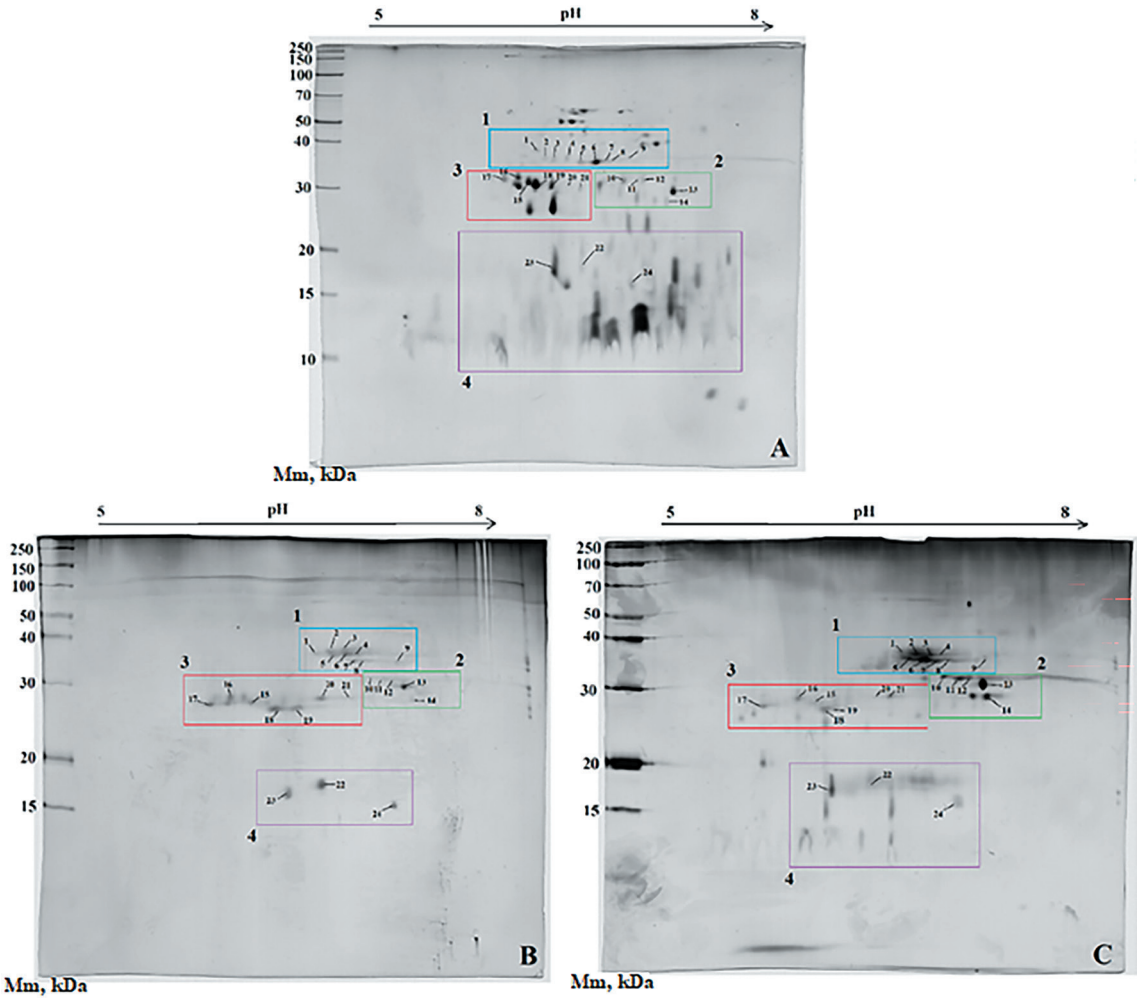

Figure 3. Two-dimensional electropherograms of PG extract (A), precipitated diafiltrate (B), and non-precipitated diafiltrate (C) obtained using 0.9% NaCl-0.5 M trehalose as extractant. Legend keys: Block 1 — marked blue; Block 2 — marked green; Block 3 — marked red; Block 4 — indicated mauve

sedimentation and exceeded the intensity values on electropherograms of the extract and DF with sedimentation by 1.46 times and 1.22 times, respectively. It is interesting to note that the proteins No. 10–12, as well as No. 14, barely noticeable in electropherograms A and B, were clearly visible in electropherogram C. The protein fraction with a MW of 29 kDa and pI 7.2 is clearly visible in the DF electropherogram without sedimentation (C) and had a coloring intensity of 17,042,702 c. u., while in the electropherogram of the extract (A) its intensity was 2,623,714, which was 6.5 times lower than its intensity in DF without sedimentation (C). In general, the electropherogram of extract (A) featured the greatest diversity of protein fractions in the studied area, and the electropherogram of DF with sedimentation featured the smallest diversity.

The following proteins may be found within this molecular weights range: chymotrypsin C [53], chymotrypsinogen B2 [54,55], member of the chymotrypsin-like family elastase 1 [28], and elastase [56]. The most expressed protein in this block, marked with number 13, presumably may correspond to chymotrypsinogen B2 (29.1 kDa, pI 7.43) [57].

In block 3 the groups of protein fractions were found that belonged to the MW range of 25–31 kDa and pI from 5.6 to 7.0. Seven protein spots were found in the electropherograms of all studied samples (fractions No. 15–21), and for the proteins 16 and 17 pI shift towards the area of 5.7–5.8 was observed in the electropherograms of the diafiltrates (B and C), compared with the electropherogram of the extract (A), where the pI values of proteins 16 and 17 were equal to 6.2 and 6.1, respectively. It is also necessary to note the shift in the molecular weights of protein fractions 15–21 in the diafiltrates by an average of 2–3 kDa to the lesser values, in comparison with the extract. In general, the intensity of staining of protein spots 15–21 was higher in the electropherogram of the extract (A) and it reached 60,599,552 c. u. (fraction 18), while in the DF electropherograms with and without sedimentation this value reached 27,059,680 c. u. (fraction 18) and 12,459,113 c. u. (fraction 17) respectively. However, it is worth paying attention to the fraction number 20, which intensity in the 2-DE extract was 770,700 c. u., which was 17.8 times less than in the 2-DE diafiltrate with sedimentation, and 3.4 times less than in the 2-DE diafiltrate without sedimentation. Comparing electropherograms B and C, one can note that block 3 in electropherogram C featured a wide variety of protein spots, on which basis it can be concluded that the sedimentation technology in the preparation of diafiltrate sample for two-dimensional electrophoresis leads to "adhesion" of some protein fractions.

Table 2. Major protein fractions observed in 2D-electropherograms

		Extract (A)		DF with sedimentation (B)		DF without sedimentation (C)	
	Fraction number	MW, kDa / pI	Volume of the spot, c. u.	MW, kDa / pI	Volume of the spot, c. u.	MW, kDa / pI	Volume of the spot, c. u.
1 block	1	39/6.4	3250480	37/6.8–6.9	2462218	37/6.6	8209223
	2	37/6.3	2078607	38/6.8–6.9	18222116	37/6.7–6.8	22283304
	3	37/6.5	6814863	38/6.9–7.0	11433116	37/6.9	18283564
	4	37/6.6	3902051	37/7.0	7775438	37/6.9–7.0	9530793
	5	35/6.7	2578116	35/6.9	9815580	36/6.7	14523944
	6	35/6.9	19726360	35/7.0	3651586	36/6.7–6.8	25627460
	7	35/6.9	2620893	34/7.1	6899868	34/6.9	11582044
	8	35/7.0	1129037	34/7.2	5317755	34/7.0	6214498
	9	35/7.1	2019942	34/7.3	1770069	36/7.3	2407177
2 block	10	31/7.2	3844677	31/7.3	7252735	31/7.2	13198519
	11	31/7.1	1728022	31/7.2	9658015	31/7.1–7.2	22687422
	12	31/7.0	5705523	31/7.1	3543859	31/7.0	12672727
	13	29/7.3–7.4	41674312	29–30/7.4	50013524	30–31/7.3	60929312
	14	28/7.4	4011557	28/7.4	18876574	29/7.3	27470042
3 block	15	31/6.3–6.4	31991576	27/6.3	10863911	28/6.1	5363564
	16	32/6.2	21427866	28/6.0	10201778	29/5.9	8694539
	17	31/6.1	10504652	27/5.8	18248104	28/5.7	12459113
	18	30–31/6.4	60599552	26/6.4–6.5	27059680	27/6.3	11679986
	19	30–31/6.5	27205454	26/6.6	25008948	27/6.3–6.4	3282697
	20	30/6.6	770700	28/6.7–6.8	13699304	29/6.5	2657022
	21	30/6.7	3634289	28/7.0	5857993	29/6.6	9958788
4 block	22	18/6.7	5988978	17/6.7–6.8	44363600	18/6.4–6.5	13464702
	23	17–18/6.5	91543784	16/6.5	4798642	17–18/6.2	28625686
	24	16/7.0	5304107	15/7.3	36110256	16/7.1	6687567

Block 3 may contain proteins such as trypsin [29] and trypsinogen [58,59]. Fraction number 20, which was most expressed in DF extracts, presumably may correspond to trypsinogen (28.2 kDa, pI 6.85).

The protein spots marked in the block 4 of the 2-DE extract (A), had molecular weight range from ~22 kDa to 10 kDa and isoelectric points from 5.4 to 7.8. In case of diafiltrate, in electropherograms B and C the molecular weights of the proteins in the block 4 ranged from 20 kDa to 10 kDa, and the isoelectric point ranged from 6.2 to 7.4. Block 4 on the electropherogram of the extract featured a wide variety of protein spots, as well as the availability of proteolytic changes in them in the area below 15 kDa. In block 4 on the electropherogram C (DF without sedimentation), protein fractions were noted within the area of MW ~19 kDa and pI 6.9–7.3, which were not observed in the electropherogram of DF with sedimentation (B). In general, in the block 4, 3 the protein fractions were found that were present in the electropherograms of all studied samples (fractions No. 22–24). The highest intensity of staining among the total protein fractions was characterized by fraction 23 on electropherogram A, having a MW of 17 kDa and pI of 6.5; the volume of this protein spot was 91,543,784 c. u., which was 20.5 times greater than in the electropherogram B and 3.2 times greater than in the electropherogram C.

The following protein fractions may be found within this molecular weight range: colipase [33], insulin [32], proglucagon [60], secretin [30], trefoil factor-2 [61], gastrin [31], cholecystokinin [62], phospholipase major isoenzyme [63].

Comparing the electropherograms in Figure 3 of the extract (A), DF with sedimentation (B) and DF without sedimentation (C) using 0.9% NaCl-1% Gly-0.1M Pro as an extractant, it was found that protein profile of the DF radically differed from the extract, and therefore it was difficult and uninformative to compare the DF electropherograms (B and C) with the electropherogram of the extract. However, it was found that both the DF electropherogram without sedimentation and the DF electropherogram with sedimentation showed fractions of the protein aggregates with MW above 50 kDa, although the membrane with a 50 kDa cutoff was used for diafiltration. The extensive protein aggregates were also observed in the alkaline area. The availability of protein aggregates gave the ground to suggest that the presence of amino acids in the extract allowed the proteins to acquire/remain in a conformation that prevented them from adhesion to each other, and after their removal during dialysis, the proteins prone to aggregation formed this area at 2-DE. During the preparation of the samples with sedimentation, it was noted that the intensity of protein fractions increased.

Comparing the protein groups in the block 1 in the electropherograms B and C, one can note a shift in the pI of the protein fractions from the range of 6.6–7.1 (electropherogram C) to the range of 7.0–7.6 (electropherogram B). The change in the molecular weights of the proteins from 49–65 kDa was also detected (electropherogram C) to 49–69 kDa (electro-

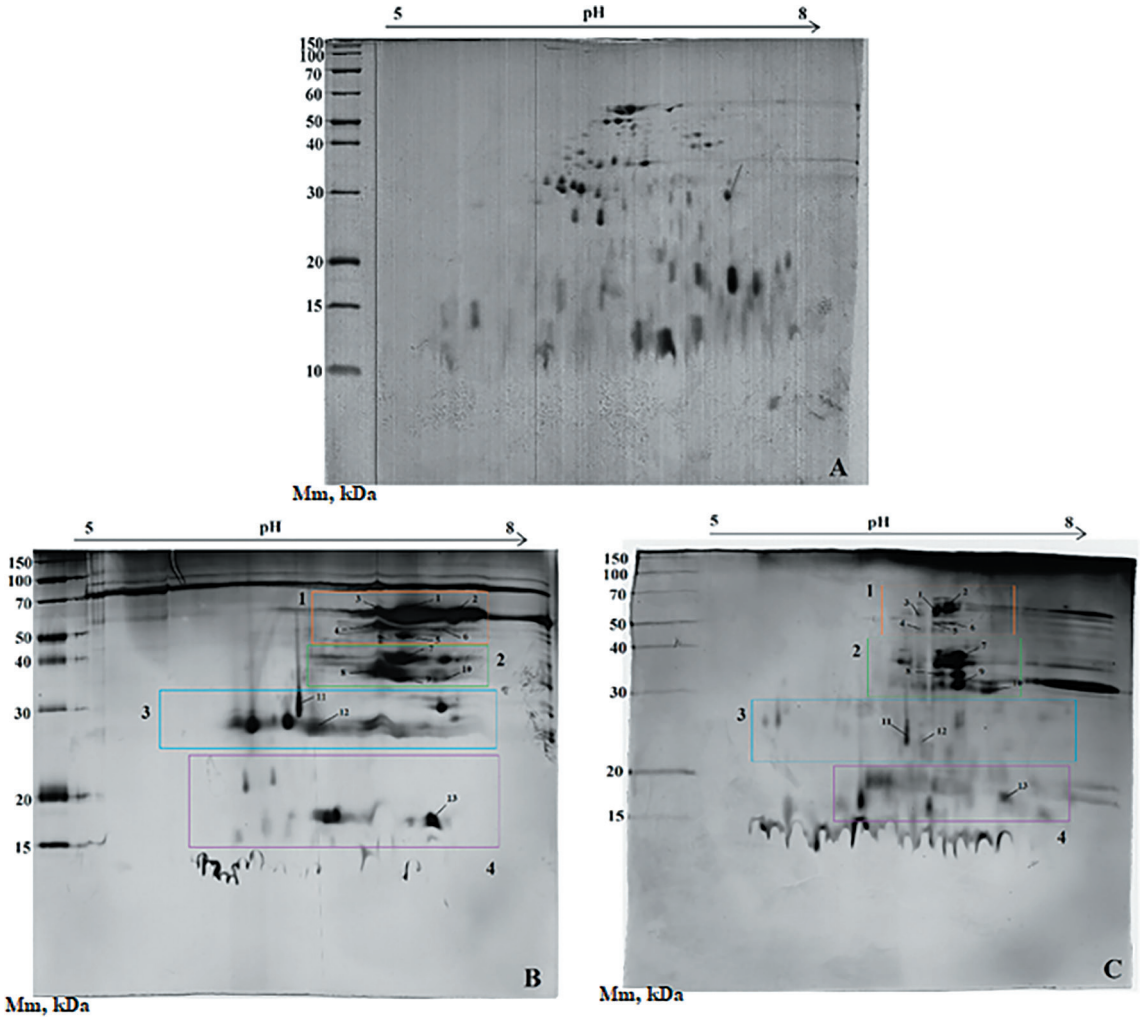

Figure 4. Two-dimensional electropherograms (2- DE) of pancreas gland extract (A), sedimented diafiltrate (B) and diafiltrate without sedimentation (C), obtained using 0.9% NaCl-1% Gly-0.1M Pro as extractant. Legend: Block 1 — marked orange; Block 2 — marked green; Block 3 — marked blue; Block 4 — indicated mauve

pherogram B). Despite the difficulty in interpreting the results due to presence of protein aggregates, 6 protein fractions were detected that were present in both electropherograms (fractions 1–6, Table 3). Fraction 1 was characterized by the highest intensity of staining on the DF electropherogram with sedimentation (B); the volume of the spot made up 151,776,770 c. u. (MW 55–69 kDa; pI 7.1–7.4), which was 19.2 times more than in the DF electropherogram without sedimentation (C). The least intensely expressed fraction in this block of proteins was the fraction No. 4 on the DF electropherogram without sedimentation (C), its volume was 799,690 c. u. (MW 49 kDa; pI 6.7), while the volume of this fraction in the DF electropherogram with sedimentation (B) comprised 17,187,413 c. u. (MW 53–60 kDa; pI 6.9–7.1), which was 21.5 times more. In this range of protein weights, the most expressed fractions may presumably be triacylglycerol lipase [64], pancreatic triacylglycerol lipase [24] and pancreatic lipase-associated protein [25].

Block 2 in the electropherograms was represented by groups of the proteins with pI 6.6–7.3 and MW 30–39 kDa (electropherogram C) and with pI 6.9–7.5 and MW 35–41 kDa (electropherogram B). Four protein fractions were found that matched both electropherograms. Block 2 on the electropherograms was represented by groups of proteins with pI 6.6–7.3 and MW 30–39 kDa (electropherogram C) and with pI 6.9–7.5 and MW 35–41 kDa (electropherogram B). Four protein fractions were found that coincided in both electropherograms (fractions No. 7–10, Table 3). Fraction No. 8 on 2-DE diafiltrate with sedimentation (B) featured the highest intensity of staining, its volume was 65,789,330 c. u., while its volume on the 2-DE diafiltrate without sedimentation (C) was 8.9 times less, and its molecular weight on the electropherogram C shifted into the range of 33–34 kDa, in comparison with electropherogram B, where its MW ranged within the area of 38–40 kDa. The fraction No. 10 was the least expressed (among the total protein fractions) on 2-DE diafiltrate with sedimentation, with MW of 37 kDa and pI 7.4–7.5, its intensity made up 26,984,492 c. u., which is 2.4 times less than for 2-DE diafiltrate without sedimentation. Presumably, the most pronounced fraction in the block 2 corresponded to phospholipase 2A (39–40 kDa) [26].

Block 3 shows groups of protein fractions within the range of MW 20–29 kDa and pI 5.5–7.8. Despite the wide variety of protein fractions available in this block in both electropherograms, there were only 2 common protein fractions (fractions No. 11 and 12). Fraction No. 11 featured

Table 3. Major protein fractions observed in 2 D electropherograms

		DF with sedimentation (B)		DF without sedimentation (C)	
	Faction number	MW, kDa / pI	Volume of the spot, c. u.	MW, kDa / pI	Volume of the spot, c. u.
1 block	1	55–69/7.1–7.4	151776770	55–65/6.8–6.9	7901400
	2	55–69/7.4–7.6	141606173	55–65/6.9–7.0	9312176
	3	60–69/6.9–7.0	24085491	55–60/6.7	3969649
	4	53–60/6.9–7.1	17187413	49/6.7	799690
	5	50/7.1–7.2	17276026	50/6.8–6.9	1935011
	6	53/7.4	4868449	50/6.9–7.0	1373070
2 block	7	39–41/7.0–7.4	56484832	35–39/6.8–6.9	47240403
	8	38–40/6.9–7.0	65789330	33–34/6.8–6.9	7359654
	9	37–40/7.1–7.2	40374312	30–31/6.9–7.1	12979550
	10	37/7.4–7.5	26984492	29–30/7.2–7.3	64556730
3 block	11	29–33/6.5–6.6	77202204	23–27/6.7	18918796
	12	27–28/6.6–6.7	55503144	21–23/6.8	2752734
4 block	13	16–17/7.3–7.4	98002590	16–17/7.4	25712488

a higher intensity of staining both in the DF electropherogram with sedimentation (B) and in the DF electropherogram without sedimentation (C). On 2-DE diafiltrate with sedimentation its molecular weight and volume made up 29–33 kDa and 77,202,204 c. u., while for 2-DE diafiltrate without sedimentation this value was 23–27 kDa and 18,918,796 c. u. The isoelectric point of this fraction was also shifted: in the case of sedimentation of the diafiltrate, the pI value accounted for 6.5, and without sedimentation it was 6.7. The lowest intensity among the total protein fractions in this block was observed in the fraction No. 12 at 2-DE diafiltrate without sedimentation (C) and amounted to 2,752,734 c. u., which was 20.2 times less than in 2-DE diafiltrate with sedimentation. Elastase is present in this range of protein weights [27], chymotrypsin-like elastase [28] and chymotrypsin [65].

Protein fractions isolated in the block 4 had a molecular weight within the range of 15–22 kDa and isoelectric points from 6.3 to 7.4. As in the case of the block 3, despite the rather large number of protein fractions in the block 4, there was only one common fraction for electropherograms B and C (fraction no. 13). Its molecular weight was the same in both electropherograms and amounted to 16–17 kDa, the isoelectric point was within the area of 7.3–7.4. The intensity of fraction No. 13 was greater at 2-DE diafiltrate with sedimentation (B) and amounted to 98,002,590 c. u., while for 2-DE diafiltrate without sedimentation (C) it amounted to 25,712,488 c. u. The most pronounced protein in this block, presumably, may be a phospholipase (16.4 kDa).

When studying the biological properties of the obtained DFs in experiments in vitro, it was found that after 6 days the pancreatic cells spread over the surface of the Petri dish the culture was represented by islet of alpha-, delta-, acinar, ductal and stellate myofibroblast-like cells (capable of switching from tranquil phenotype to an activated phenotype and back), the latter were more numerous in the control Petri dish (Figure 4). After 9 days, in the control dish the predominantly contaminating substances were observed, it was fibroblast-like cells and a small quantity of spindle-shaped cells.

It was noted that when the studied samples were added into the medium, on the 6th day the islet cells showed good adhesion and spreading over the surface, forming a denser monolayer of cells.

It is interesting to note that when the samples of DF 0.9% NaCl-0.5 M trehalose and DF 0.9% NaCl-1% Gly-0.1 M Pro were added into the medium, the cells retained their rounded shape. It was particularly expressed when added to the medium DF 0.9% NaCl-0.5 M trehalose, despite the fact that, in accordance with [66], primary isolated pancreatic cells grown on 2D substrates usually possess flatter and elongated morphology. In experimental dishes the cells formed rosette-shaped micro-communities, in dishes with a nutrient medium with DF 0.9% NaCl-0.5 M trehalose added the beta cells were detected taking on the typical morphology of polarized polyhedron.

In result of studying the biological properties obtained by DF in experiments *in vitro* on the primary isolated mice pancreatic cells, it was found that when the studied samples were added to the medium, the islet cells showed good adhesion and spread over the surface on the 6th and 9th days, forming a denser monolayer of cells (Figure 4). After 9 days, in the control dish the predominantly contaminating substances were observed, it was fibroblast-like cells and a small quantity of spindle-shaped cells.

In experimental dishes cells formed rosette-shaped micro-communities, in dishes with a nutrient medium with DF 0.9% NaCl-0.5 M trehalos e added, the beta cells were detected taking on the typical morphology of a polarized polyhedron.

The results of recording the viability and number of the cells on the 9th day are presented below in the Table 4.

Table 4. Impact of DF 0.9% NaCl-0.5 M trehalose and DF 0.9% NaCl-1% Gly-0.1 M Pro at a concentration of 100 ng /ml on the number and viability of the pancreatic cells

	Concentration of living cells (cells /ml)	Survival rate (%)	Average cell size (µm)
Control	3.68×10^5	47.4	16.6
Gly	3.17×10^5	85.5	15.5
Treg	2.88×10^5	73.9	16.8

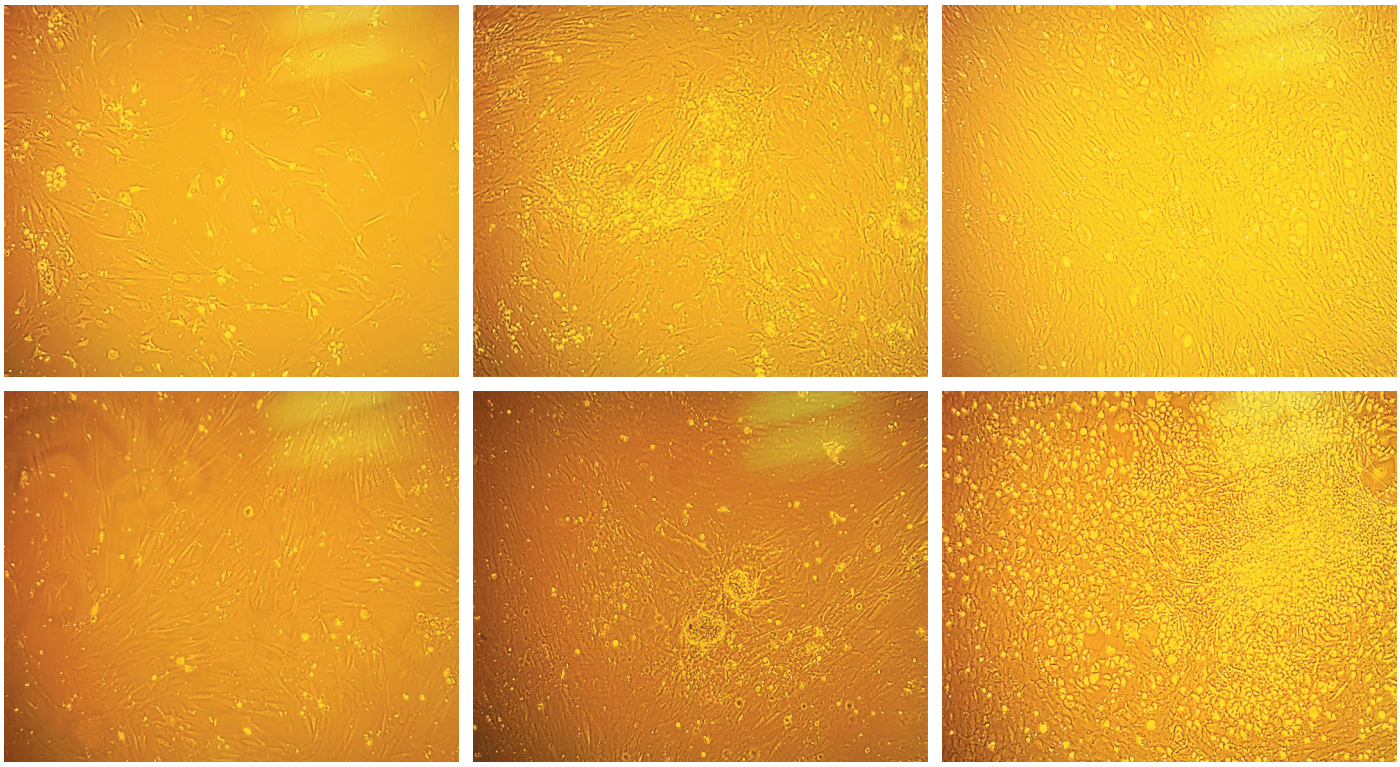

Figure 4. Culture of pancreatic cells on a Petri dish: from above — after 6 days, from below — after 9 days. Inverted microscope, magnification ×100

The high concentration of living cells of a larger volume found in the control sample, as well as their high survival rate, is related to the predominance of fibroblast-like cells in the culture, while there is a smaller number and lower survival rate of cells grown in a medium with DF 0.9% NaCl-0.5 M trehalose added is related to the predominance of islet cells, possibly beta cells, in the medium. This is quite important due to the fact that beta cells are particularly get affected by hypoxia, showing reduced viability and loss of insulin secretory capacity soon after isolation and cultivation on a flat 2D substrate [66].

Conclusion

The methodology for separation of the protein-peptide mixtures, obtained from pigs' pancreas glands using 0.9% NaCl-1% Gly-0.1M Pro as an extractant, allowed extracting more proteins than when using 0.9% NaCl-0.5 M trehalose. Press-type ultrafiltration followed by dialysis was proved inefficient in both approaches. However, when analyzing two-dimensional electropherograms, it was found that during the diafiltration technology using 0.9% NaCl-0.5 M trehalose as an extractant, the proteomic distribution of diafiltrates on 2-DE in the area of less than 50 kDa (protein cutoff threshold for diafiltration) corresponded to the proteomic distribution in the extract. The application of 0.9% NaCl-1% Gly-0.1M Pro contributed to significantly higher yield of the proteins into the diafiltrate, however on 2D-EF there were many protein aggregates noted, including those with MW above 50 kDa, though during diafiltration a membrane with a cutoff of 50 kDa was used. The availability of the protein aggregates suggests that the presence of the amino acids in the extract and diafiltrate allowed the proteins to acquire/remain in a conformation that prevented them from sticking to each other and after their removal during dialysis, the proteins aggregated to each other again. When using 0.9% NaCl-0.5 M trehalose as an extractant, the protein fractions of annexin A2, chymotrypsinogen B2 and trypsinogen were most intensely expressed, and the use of 0.9% NaCl-1% Gly-0.1M Pro contributed to the highest yield of the following fractions: triacylglycerol lipase, pancreatic triacylglycerol lipase — the protein associated with pancreatic lipase and phospholipase.

The results of experiments *in vitro* showed that diafiltrates of pigs' pancreas glands obtained with addition of 0.9% NaCl-0.5 M trehalose and 0.9% NaCl-1% Gly-0.1 M Pro into the nutrient medium, contribute to the preservation of the function and rate of survival of islet cells of mice pancreas gland. Diafiltrate obtained with trehalose promoted the forming of rosette-shaped microcommunities, preservation of the round shape of islet cells and beta cells, which may be explained by the fact that after removal of trehalose by dialysis from a protein fraction with a molecular weight of less than 50 kDa, its residual quantities were still sufficient to prevent the proteins aggregation and, as a consequence, the extracted proteins preserve their biological activity.

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DEVELOPMENT OF A MOBILE APPLICATION FOR RAPID DETECTION OF MEAT FRESHNESS USING DEEP LEARNING

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Keywords: *Meat quality, rapid detection, deep learning, red meat quality, image processing, flutter, Android*

Abstract
The freshness or spoilage of meat is critical in terms of meat color and quality criteria. Detecting the condition of the meat is important not only for consumers but also for the processing of the meat itself. Meat quality is influenced by various pre-slaughter factors including housing conditions, diet, age, genetic background, environmental temperature, and stress factors. Additionally, spoilage can occur due to the slaughtering process, though post-slaughter spoilage is more frequent and has a stronger correlation with post-slaughter factors. The primary indicator of meat quality is the pH value, which can be high or low. Variations in pH values can lead to adverse effects in the final product such as color defects, microbial issues, short shelf life, reduced quality, and consumer complaints. Many of these characteristics are visible components of quality. This study aimed to develop a mobile application using deep learning-based image processing techniques for the rapid detection of freshness. The attributes of the source and the targeted predictions were found satisfactory, indicating that further advancements could be made in developing future versions of the application.

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Introduction

Red meat serves as a crucial source of animal protein for a healthy human diet [1]. In Turkey, pork is not consumed, so the need for red meat is met through butcher cattle and meat based dishes are popular in this country [2]. Despite rising meat prices due to a decreasing cattle population, beef is still one of the most important dietary sources. Also meat products such meatball [3], fermented sausage [4] or pastirma [5] are the most consumed meat products. For meat and meat products, quality is very important and changes in beef quality depend on several post-slaughter factors. Additionally, pre-slaughter stress, improper slaughtering techniques, and genetic characteristics also impact meat quality. Meat quality is closely related to alterations in the structure of meat proteins. Therefore, understanding the post-mortem changes in meat is highly significant for enhancing the quality of meat and meat products [6].

The procedures applied to butcher animals before slaughter play a critical role in determining the hygienic and technological quality of the carcass both during and after the slaughter process. Pre-slaughter conditions are established from the time cattle are loaded at the farm gate until the moment of slaughter. These conditions include factors such as breed and pre-transport feeding, transport distance and duration, loading density, holding methods in abattoir pens, holding duration, fasting duration, and post-transport mobility within the pen.

Following the slaughtering process, metabolic reactions continue in the muscles. As circulation ceases, the muscles try to replenish the necessary energy using glycogen stored within them. If there is an insufficient amount of glycogen in the muscles or if post-mortem energy cannot be provided due to several reasons, adequate levels of lactic acid may not accumulate in the muscles. Consequently, metabolic reactions may not form as expected, and the quality of the meat may not reach the desired level [7].

Prolonged activity of animal muscles under inappropriate conditions such as stress, without adequate rest before slaughter, results in a decrease in glycogen reserves. This low level of glycogen reserve leads to the formation of only a small amount of lactic acid. Consequently, meat with a pH value of 6.0 becomes dark, firm, and dry (DFD: Dark, Firm, Dry) as reported by Young et al. [8]. In DFD meats, the high pH value increases the risk of microbial growth (making them less durable), enhances water-holding capacity, and darkens the color [9]. Conversely, Pale, Soft, Exudative (PSE) meat is often a result of stress accelerating glycolysis and causing a faster-than-normal drop in pH value [10]. Under the influence of these stress factors, the pH value can drop rapidly to 5.3 within 1–1.5 hours, and an acidic rigor-mortis develops. The drop in muscle pH and the normal temperature of the muscle at the time of slaughter cause certain proteins, especially myosin, to denature. In PSE meats, the color is pale, the texture is soft, the surface is watery, and the water-holding capacity is low [11]. These types of meats are primarily utilized in the production of cured raw meat and fermented meat products. Within the two hours after slaughter, the muscle pH typically drops to 5.6 and even

to as low as 5.2 [9]. However, illumination conditions or storage also effects on surface [12], but these factor are controllable after slaughter.

Some spectroscopic methods are developed to classify these disordered meat sources. In a research, the factors involved in developing Vis/NIR spectroscopy models to differentiate between PSE (Pale, Soft, Exudative), DFD (Dark, Firm, Dry), and normal chicken breast meat were evaluated [13]. To further explore the differences between PSE, DFD, and normal meat, various studies have been conducted using different deep learning techniques [14]. It was also suggested that the deep learning approach can be a fast and innovative way for the assessment of chicken meat quality [15]. It's important to understand some definition such as machine learning, convolutional neural networks, MobileNet and transfer learning models. Machine learning is a kind of artificial intelligence (AI) and it has been focused on developing algorithms based on data patterns [16]. It also has historical data relationships for that purpose [17]. This approach is fundamentally like the way humans learn. This process increases accuracy by using data and algorithms [18]. Convolutional Neural Networks (CNNs) are a class of deep neural networks. This class is widely used in various fields such as image processing. CNNs have been used in various fields such as fruit classification [19]. MobileNet is a type of architecture designed especially for mobile and embedded vision applications [20].

MobileNet provides sufficient performance on low-volume devices [21,22]. In the context of image classification, MobileNet had a good performance especially with lower training time [23]. Transfer learning is a machine learning technique where a model trained on one task is re-purposed on a second related task [24]. In the context of MobileNet, transfer learning has been widely applied to leverage the pre-trained MobileNet architecture for various tasks. Li et al. [25] performed transfer learning on lightweight CNNs, including MobileNet, for plant disease leaf detection on cell phones, demonstrating the versatility of MobileNet in agricultural applications.

The Google Teachable Machine platform allows training and testing machine learning models using the transfer learning technique [26]. Google Teachable Machine uses a pre-trained MobileNet model and transfer learning. The three basic parameters of a convolutional neural network (CNN) are epoch, batch size, and learning rate [27]. An epoch refers to one complete pass with entire training dataset. The batch size refers to the number of training examples utilized in one iteration of the gradient descent algorithm. The learning rate is a parameter that controls the magnitude of the updates to the model's weights during training. These parameters are crucial for getting optimal results in CNN training [27].

ResNet-50, developed by Microsoft Research Asia, is a convolutional neural network (CNN) architecture that addresses the gradient vanishing problem by introducing residual connections [28]. It is a deep neural network that is part of the ResNet family, known for its ability to perform well with deeper networks due to its residual connections [29]. ResNet-50 is specifically a 50-layer deep CNN architecture [30,31]. ResNet-50 has been used in some applications such as object detection and classification.

The aim of this study is to understand the practical usability of this model with a mobile application by developing a deep learning-based model in which meat defects can be displayed live and in real time using Google Teachable Machine. Analyzes were also performed with RESNET-50 to verify the results obtained with Google Teachable Machine.

Objects and methods

Mobile application design

The process of classifying meats through a mobile application has three stages. Initially, the device's camera captures an image and transmits it to a previously trained artificial intelligence model. Next, the trained model analyzes the image and outputs a classification, indicating whether the meat is fresh or spoiled. Finally, based on these outputs, the freshness or spoilage status of the meat is displayed on the screen. A flowchart detailing these stages is provided in Figure 1.

Dataset

In this study, the "Meat Quality Assessment Dataset" prepared by Ulucan et al. [32] was utilized. The dataset includes images of meat taken every two minutes, with concurrent spoilage assessments conducted by an expert, classified according to seven parameters. These parameters are date and time, ambient temperature, product temperature, color change, brightness, odor status, and regional or complete spoilage [32]. The dataset contains 948 images of fresh meat and 948 images of spoiled meat. To enrich this dataset, various augmentation techniques were applied, including angular rotation (15°, 45°, 60°, 75°, 90°, 135°, 180°, 225°, 270°, 315°), flipping (Horizontal, Vertical), scaling down (50%), scaling up (150%), noise addition (Salt and Pepper, Gaussian), and filtering (Gaussian, Median, Mode). Following these processes, a total of 18,960 images per category were obtained, and screenshots of the previews are provided in Figures 2, 3, and 4.

Figure 1. Process stages for meat classification in a mobile application

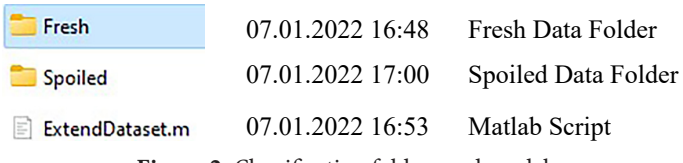

Figure 2. Classification folders and model

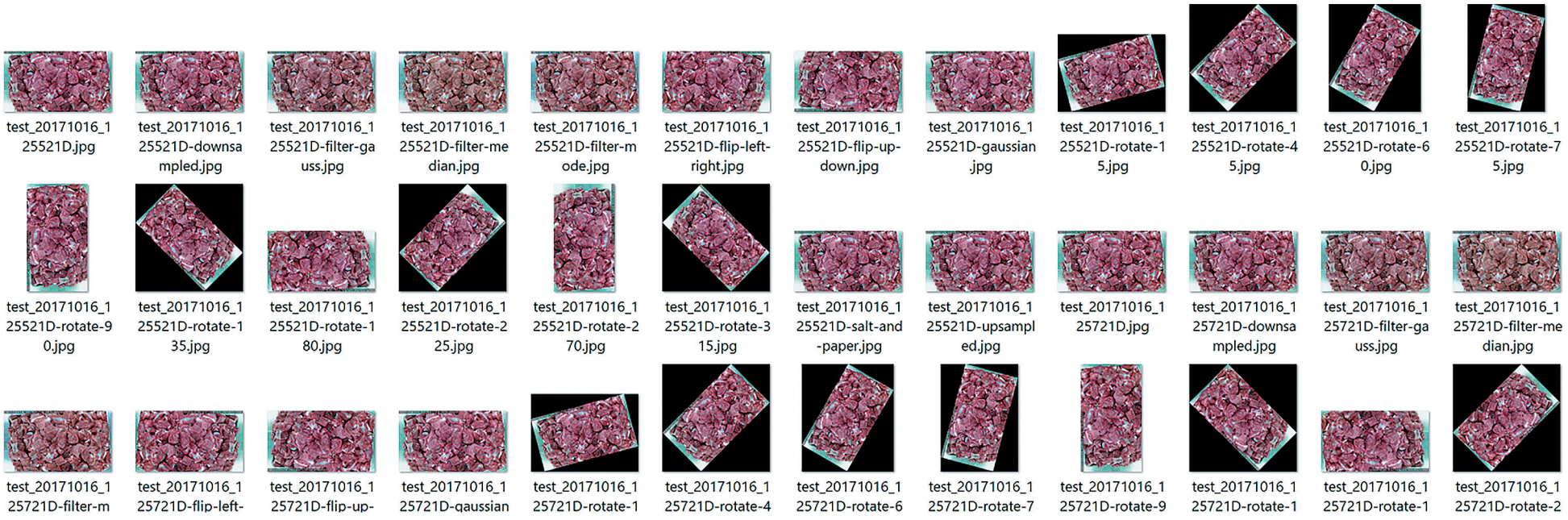

Figure 3. Images of fresh meats with processing algorithms

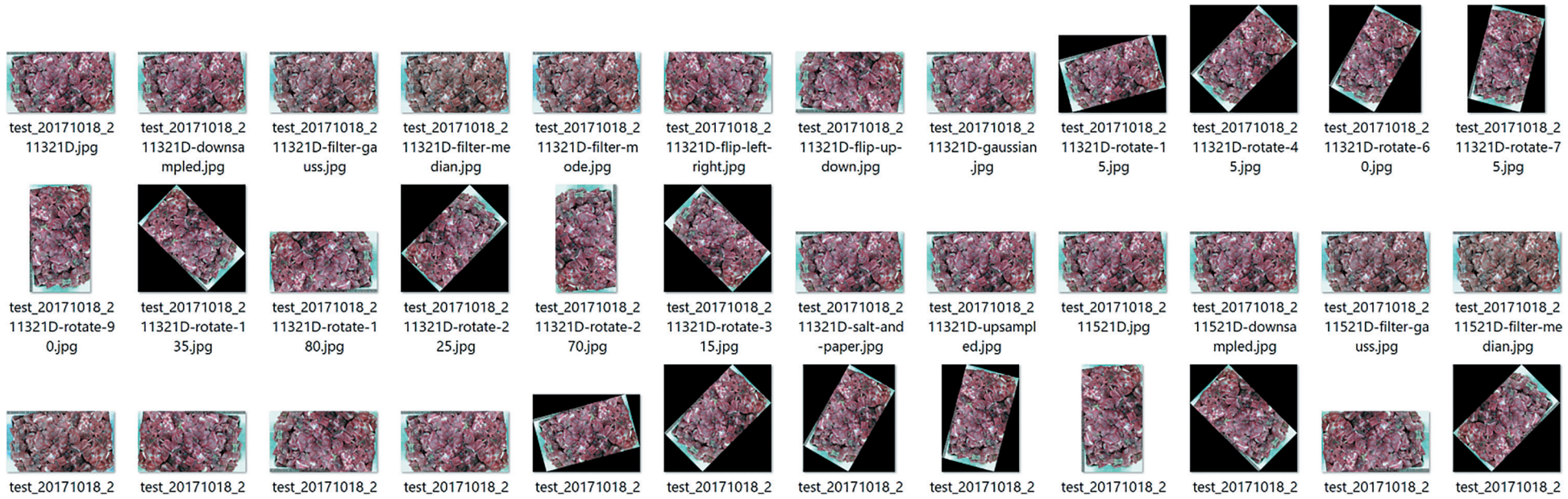

Figure 4. Images of spoiled meats with processing algorithms

Implementation of models for mobile application

For integrating a machine learning model into an Android application, the machine learning model developed using Google Teachable Machine was integrated into an Android application. A quantized model was created using the model.tflite and label.txt files.

Results and discussion

Managing Google Teachable Machine

Due to the restriction by the Teachable Machine that allows a maximum of 10,000 samples per category, 10,000 random samples from each class were selected for the study. These samples were divided into training and testing datasets, with 8,500 samples (85%) used for training and 1,500 samples (15%) reserved for testing. The accuracy rates for each class after training can be seen in Table 1.

Table 1. The results of the accuracy per class

Class	Accuracy	#Samples
Fresh	1.00	1,500
Spoiled	1.00	1,500

The model's learning process details, such as the total number of epochs, batch size, learning rate, and dataset design for inputs and outputs, are illustrated in Figure 5. This structured approach helps in comprehensively understanding how the model was trained and how it performs across different parameters and dataset characteristics.

Testing of models

The confusion matrix for the model is presented in Figure 6. This matrix helps in visualizing the accuracy of the model by showing the number of correct and incorrect predictions made for each class.

Figure 7 displays the loss analysis for each epoch. Thus, it is shown how the model's prediction error decreases over time as it learns from the training data.

Finally, Figure 8 presents a more detailed or different aspect than those shown in Figure 9. This is indicative of metrics such as precision, recall, or F1 scores that provide a more comprehensive assessment.

In the scenario described, the model showcases an optimal training performance, as indicated by the absence of overfitting or underfitting in the graphs. The accuracy analysis per epoch had a score of 1.00, which is indicator of its predictive capability. Furthermore, from the second epoch onwards, the training model appears to stabilize, maintaining consistent accuracy values without any fluctuations.

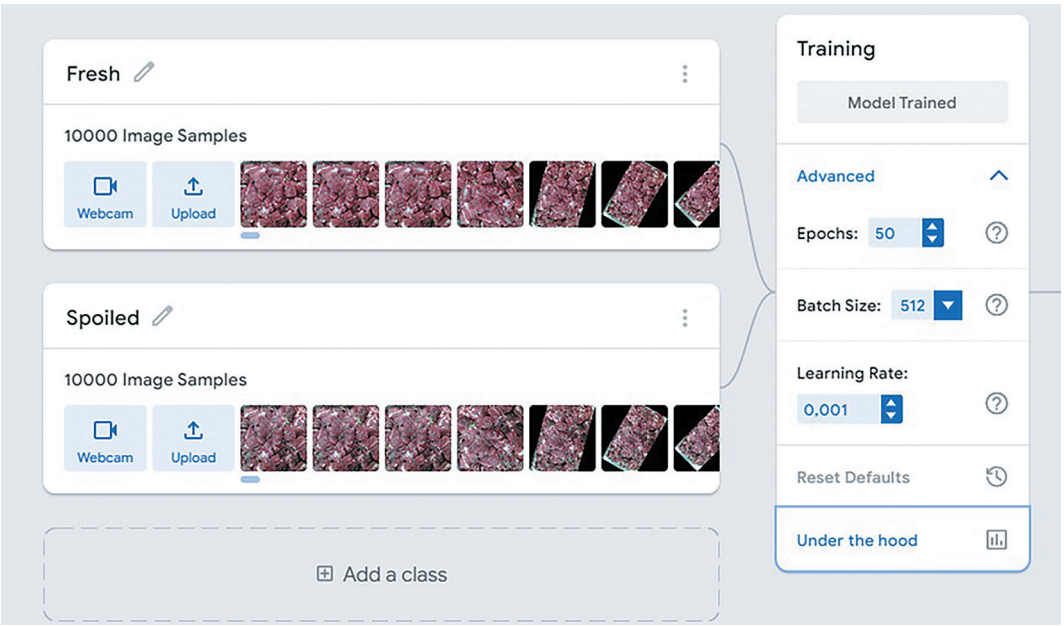

Figure 5. Model parameters

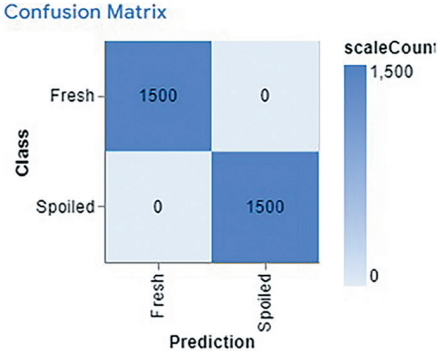

Figure 6. Confusion Matrix

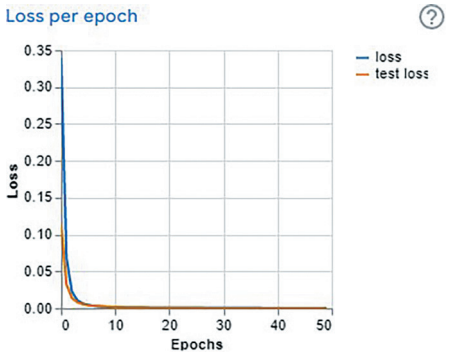

Figure 7. Loss per epoch

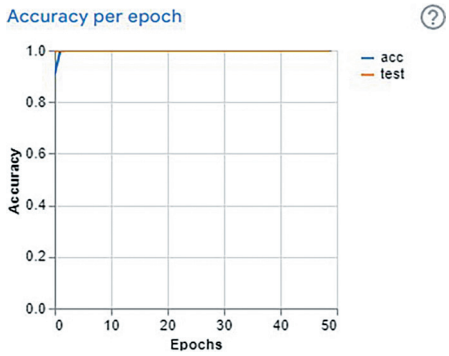

Figure 8. Accuracy per epoch

Results indicates a well-trained model. This is ideal in machine learning as it suggests that the model not only learns the patterns in the training data but also effectively applies this knowledge to unseen data, which is critical for practical applications.

System assessment

During the evaluation phase of this system, the black box testing method is used. This approach evaluates the system functionalities without knowing the internal workings of the application, which mimics how end-users would interact with the system.

Figures 9, 10, and 11 show the test images (fresh and spoiled) processed in Google's Teachable Machine.

Figure 9 presents the result of the test classification on an image of fresh meat. This would demonstrate how accurately the system can identify meat that is still fresh and safe for consumption based on its trained model.

Figure 10 displays the results of test classifications on images of spoiled meat. This is crucial for determining the system's effectiveness in correctly identifying meat that is no longer suitable for consumption, thus avoiding potential health risks.

Figure 11 contains screenshots from the mobile application developed for this testing. These images provide insight into how the application presents the classification results to the user, offering a user-friendly interface that displays whether the meat is fresh or spoiled based on the image captured by the camera sensor on an Android device.

Such evaluations and visual presentations in the testing phase are essential for validating the reliability and usability of the machine learning model and the overall system. They help to ensure that the application performs well in practical scenarios, which is key to user satisfaction and safety.

ResNet-50's unique architecture, which includes residual connections and a bottleneck structure, enables it to effectively address the challenges associated with training deep neural networks. Resnet-50 was used to validate Google Teachable Machine results. The training progress of Resnet-50 is given in Figure 12 and the results of Resnet-50 are given in Table 2.

Table 2. Validation accuracy of the proposed model

Parameter	Result
Validation accuracy	99.39%
Training finished	Max epochs completed
Training elapsed time	236 min 34 sec
Training epoch cycle	10 of 10
Iteration	9480 of 9480
Iteration per epoch	948
Max Iteration	9480
Validation Frequency	30 iterations
Hardware resource	Single GPU
Learning rate schedule	Constant
Learning rate	0.0001

Precision-Recall curve and ROC curve of ResNet-50 are also given in Figure 13 below.

Confusion matrix of ResNet-50 is given in Figure 14.

The results obtained from Google Teachable Machine and RESNET-50 are presented in Table 3, showcasing the precision, recall, and F1-score for the classes "Fresh" and "Spoiled".

Table 3. Precision, recall and F1-score results of the classes (Fresh and Spoiled)

Class	Precision	Recall	F1-Score
Fresh	0,9952	0,9926	0,9939
Spoiled	0,9926	0,9952	0,9939

The analysis demonstrates that models exhibit exceptionally high-performance metrics across both classes. The precision and recall values for both "Fresh" and "Spoiled"

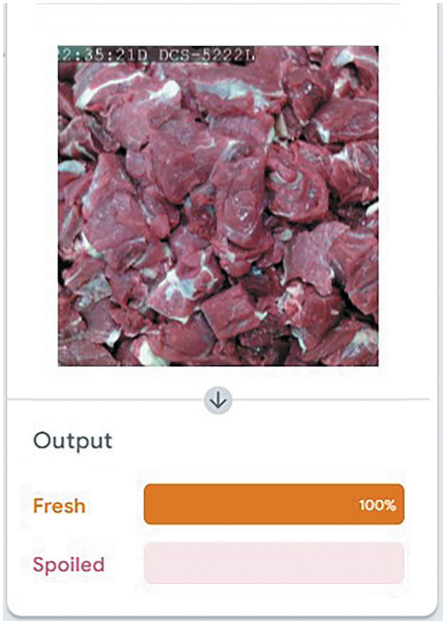

Figure 9. The result of the test classification on an image of fresh meat

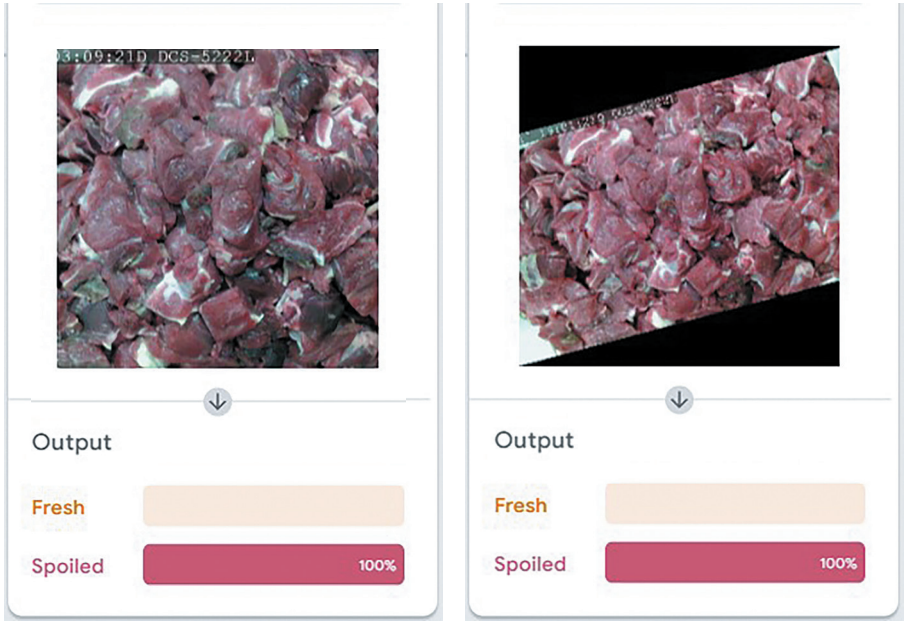

Figure 10.
The results of test classifications on images of spoiled meat

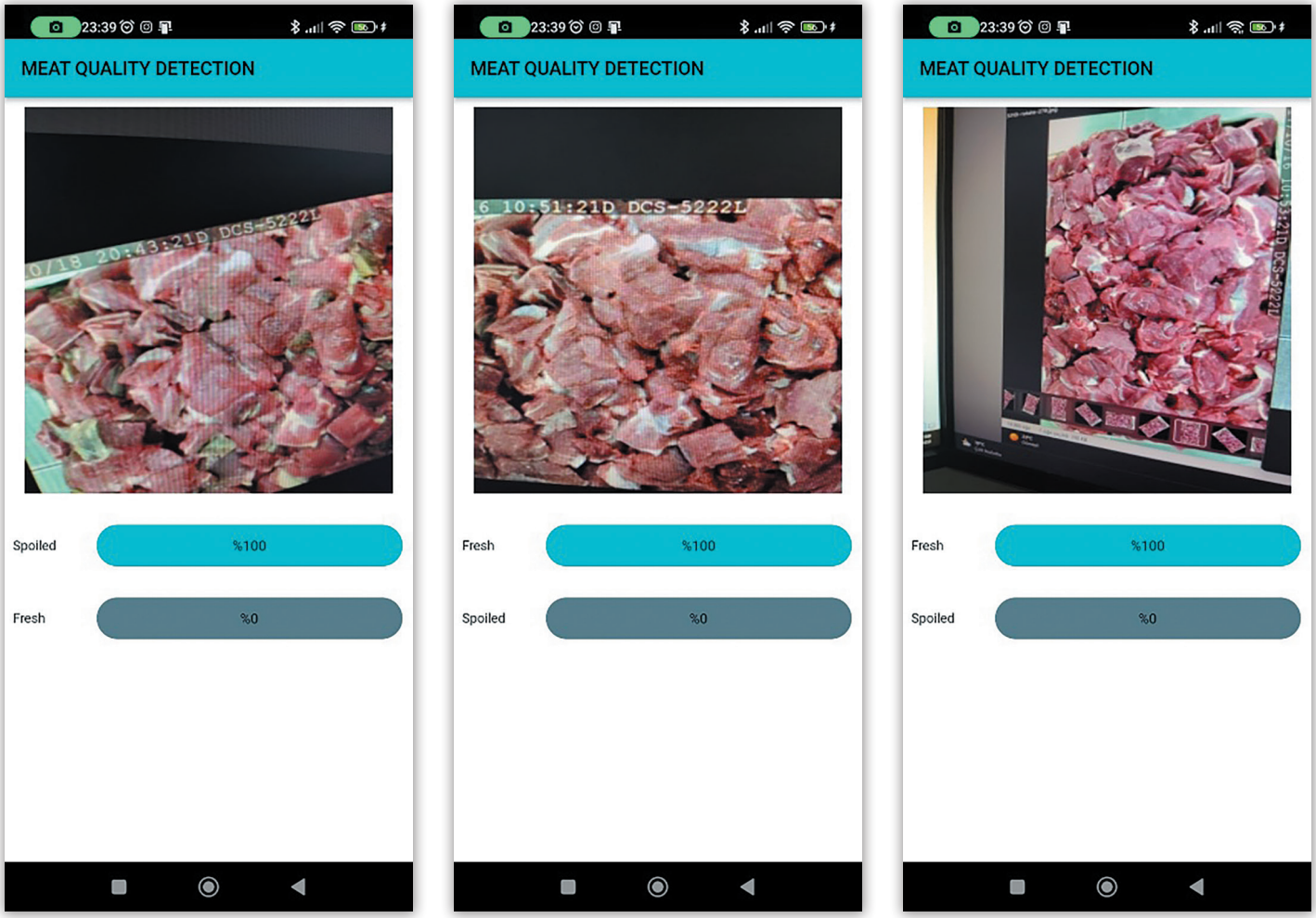

Figure 11. Screenshots from the mobile application developed for this testing

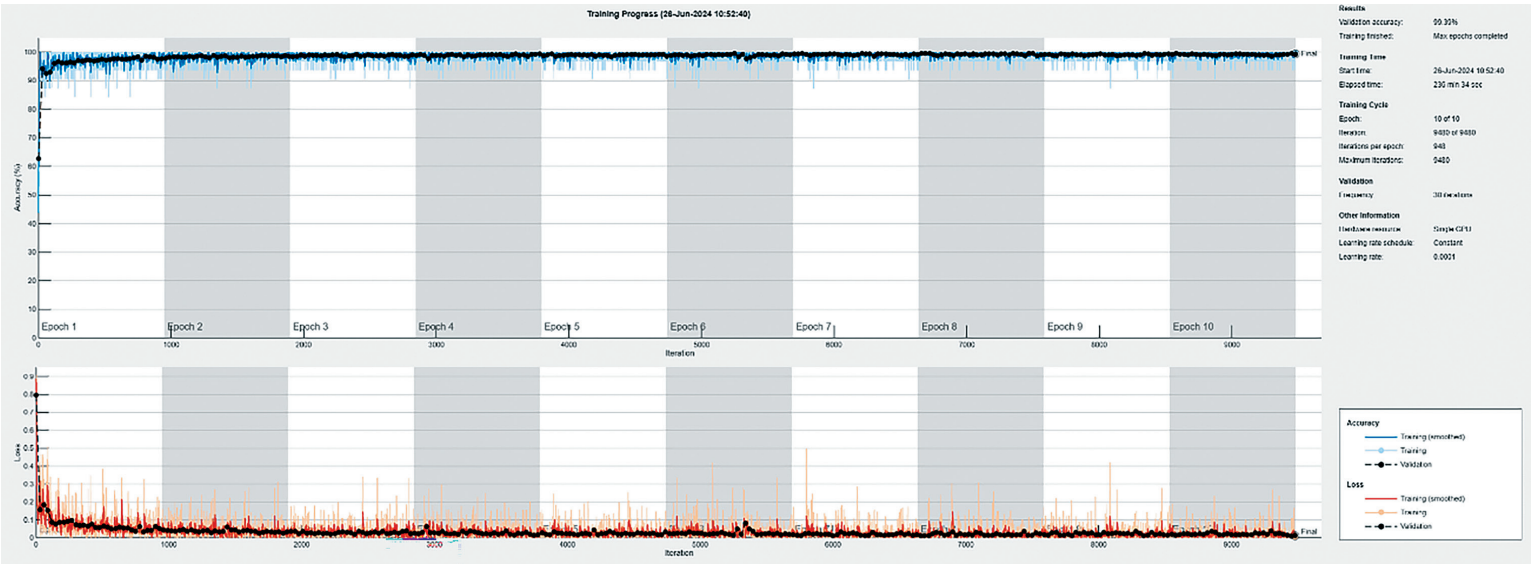

Figure 12. The Training progress of Resnet-50

categories are consistently above 0.99, indicating a highly accurate classification capability. Specifically, the precision of the "Fresh" class is 0.9952, with a recall of 0.9926, resulting in an F1-score of 0.9939. Conversely, the "Spoiled" class shows a precision of 0.9926, a recall of 0.9952, and an F1-score of 0.9939. These metrics reflect a balanced and robust classification performance, demonstrating the effectiveness of the deep learning models in distinguishing between fresh and spoiled meat with minimal misclassification.

Throughout the development process, Flutter was used to build the application, allowing for testing across different cameras and environments. This approach demonstrated the flexibility and adaptability of the application under various conditions.

The research confirmed that Android smartphone devices can effectively employ a trained dataset and model tailored for specific detection goals. By using Google's Teachable Machine, a machine learning model could be

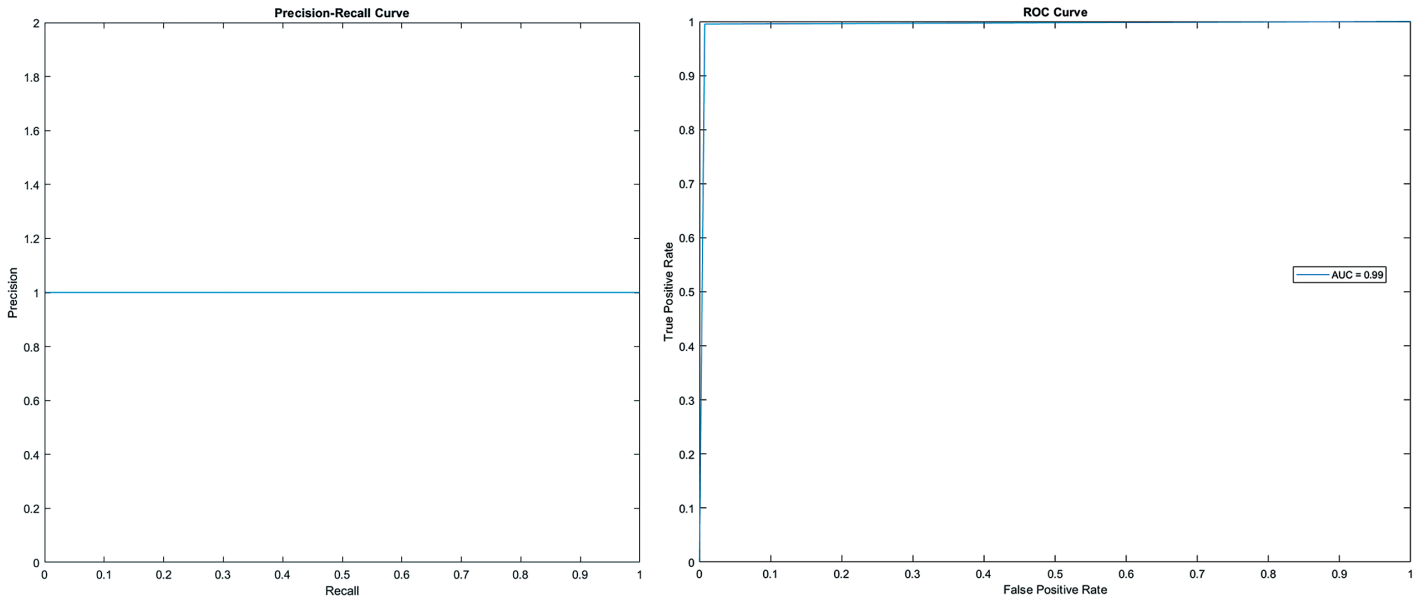

Figure 13. (a) Precision-Recall Curve, (b) ROC Curve

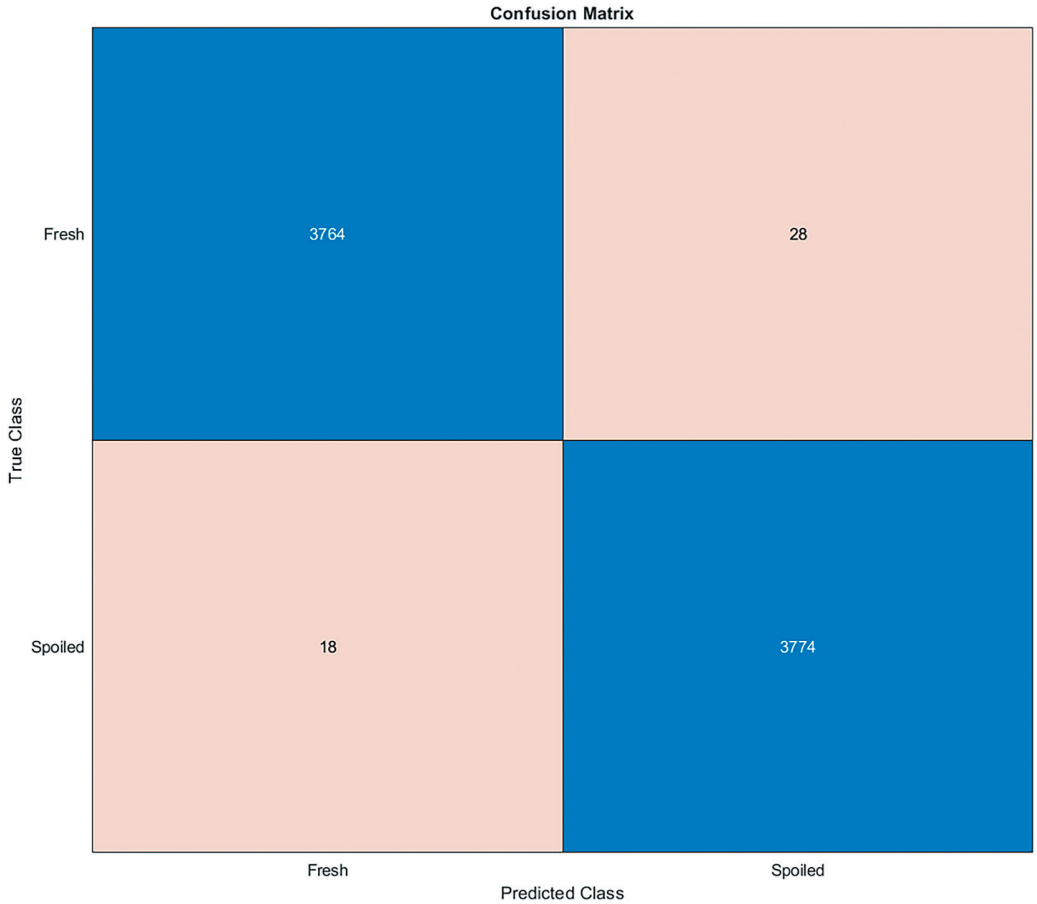

Figure 14. Confusion Matrix of ResNet-50

implemented swiftly and with minimal resource expenditures. This capability is crucial for quickly reaching targeted outcomes and addressing significant issues in the industry through further advanced research.

Conclusion

In this study, an artificial intelligence application was developed using deep learning to determine whether beef was fresh or spoiled. The results were highly satisfactory, showing extremely high accuracy, sensitivity, and precision rates, with the model created using Google's Teachable Machine achieving up to 100% in these metrics. However, it was noted that the accuracy could decrease depending on the level of lighting in the detection area. This study illustrates the potential of integrating accessible AI technologies like Google's Teachable Machine into mobile applications, providing powerful tools for industry applications where quick and accurate classification of

product quality can significantly impact health and business outcomes.

The deep learning-based mobile application for quick meat freshness detection offers several advantages, such as better meat quality evaluation and increased consumer safety. By incorporating MLOps practices, the model could be able to learn from new images continuously, which would increase accuracy and decrease bias especially in difficult lighting conditions and increase its effectiveness even further. Adding a variety of datasets will also guarantee strong performance in a range of situations. Maintaining high accuracy and adaptability through regular model updates and retraining will eventually improve user experiences and application trust.

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EXERCISING OF INTEGRATED APPROACH FOR THE SPECIALISED MEAT PRODUCTS DEVELOPMENT

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Abstract
The principles of the specialized food products development differ from the traditional technologies, which require the integrated approach to their creation, taking into consideration the specified properties, purpose and type of the food product. This article describes the general algorithm for developing the specialized dietary therapeutic and prophylactic nutrition meat products, and demonstrates the implementation of the individual stages of the algorithm on the example of developing the food products for people who suffer from the most socially significant diseases like diabetes and CVD. The algorithm of methodology is a sequence of stages executed during the product development and a description of their content and practical implementation. The modern approaches to creation of healthy food products for people with socially significant diseases, the recommendations of the World Health Organization based on the analysis of scientific literature posted in open sources and publicly available databases were used as the material of the research, as well as the results of our own studies in the field of technologies for dietary therapeutic and prophylactic nutrition meat products. The specialized meat products for therapeutic and prophylactic nutrition, information about their ingredient composition, nutritional value, results of preclinical and clinical trials were used as the objects of the study. The stages of product creation include medical and biological substantiation of the composition, designing of a virtual model of the food product, technology development, evaluation of the safety and efficiency of the resulting product taking into consideration the technological impact, preclinical and clinical evaluation. The study showed the difference in the approaches to the dietary meat products development depending on the purpose — whether its therapeutic nutrition or prophylactic nutrition. The developed methodology can be used as a tool that provides for scientifically justified development of the specialized meat products and substantiation of their efficiency.

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Introduction

According to data of WHO the healthy nutrition is one of the factors that helps reducing the risk of spreading of non-communicable diseases.

Recently an obvious trend has appeared towards the vigorous development of food technologies in various countries, which technologies can be used for prevention of chronic non-communicable diseases. This has also led to an increase of the society's demand for healthy food products [1].

The researches of many foreign authors have shown the approaches to creation of healthy food products for people who suffer from the socially significant diseases (diabetes, cardiovascular diseases (CVD)).

The researchers Villaño et al. [2] consider that the methodology for specialized food development (SFD) must differ from the principles of the traditional products developing. This is necessary to ensure the stability of functional ingredients in the food matrix and the preservation of bioactive components through the food processing, storage, digestion and absorption [2].

The literature analysis showed that the researches of many authors are focused on using the methods to reduce the risk of disease only per the one critically significant parameter, characteristic for this particular pathology.

Bolger and al [3] used the various methods of adding flaxseed oil for increasing the content and bioavailability of alpha-linolenic acid in the chicken sausages. The results of their studies showed that alpha-linolenic acid in lyophilized encapsulated forms turned to be the most bioavailable, and can be used to fortify the food products of targeted action [3].

Many studies have noted the efficiency of various bioactive compounds in the treatment of cardiovascular diseases (CVD) and diabetes. Jeevarathinam G. in his work has presented the modern methods of food processing: macromolecular method (by adding fiber and fortifying with protein), thermal (temperature nodes selection), non-

thermal (ultrasound, high pressure, pulsed electric field, microwaves, irradiation, microencapsulation, extrusion and fermentation methods) to reduce the glycemic index (GI), which contributes to increasing the blood sugar level. The used approaches are aimed to changing the macromolecular structure of carbohydrates by influencing their digestion and absorption rate, and subsequently changing the GI of the resulting food product [4].

In the works of Hernandez et al [5] and Zhu et al [6] the authors found that bioactive peptides obtained from legumes can be used to fortify food products for cardiovascular diseases prevention.

Flores-Medellín et al [7] studied the effects of fermented phenolic compounds and protein hydrolysates of black kidney beans (*Phaseolus vulgaris* L.) obtained by solid-state fermentation (SSF) on the markers associated with obesity and diabetes of the 2nd type. The results showed that this method of processing significantly improves the yield of bioactive compounds from the food matrix, increases the antioxidant and biological potential of plain kidney beans, and is very promising for obtaining the functional ingredients rich in bioactive compounds that can be used in specialized food products for diabetes prevention [7].

Yakubu [8] has run *in vivo studies* and has proven the antidiabetic properties of bitter gourd (colocynth) seed protein hydrolysate. Zhou et al. [9] discovered an α-amylase inhibitory peptide extracted from quinoa protein hydrolysate, which property proves its potential as diabetes curative substance. In the article [10], the authors analyzed the role of glucuronic acid metabolites of phenolic acids in the glucose metabolism, providing the concepts of the new therapeutic targets. Tang et al. [11] drew attention to using of marine fucosyl polysaccharides to keep blood glucose levels under control and mitigate the complications caused by hyperglycemia. Various cereals, legumes and tuber crops also contribute to reducing the GI of products. For example, adding mung beans, chickpeas, green peas and rajma beans into the recipes reduced starch digestion rate and reduced glycemic index of noodles [12,13,14,15].

In Russia food products developed for disease prevention and nutritional support of people in hospital settings are classified as specialized food products and are covered by the technical regulations TR CU027/2012[1]. All scientific researches in this area are aimed to creation of new types of food products that feature their distinctive physiological efficiency.

In the case of developing of food for nutritional support of people, special attention is focused on satisfying the physiological needs of the human body for nutrients and energy, taking into consideration the mechanisms of disease development, absence of allergic reactions among the patients, the effect of the food product on tolerance and biochemical parameters of the blood (metabolism of proteins, carbohydrates, fats, vitamin and mineral status), that prove the efficiency of disease treatment.

In the case of formulation of dietary prophylactic nutrition food assigned to reduce the risk of human diseases, the composition of the product being created should be targeted to the pathogenetic mechanisms of disease development. For example, for CVD prevention the obligatory condition of the food being composed is its cardioprotective and antiatherogenic nature. The indirect markers associated with CVD should be studied thoroughly to study the efficiency of the product [16]. They should also be aimed at increasing the protective and antioxidant functions of the human body, regulating the processes of biotransformation of foreign compounds and their removal from the body, thus reducing the risk of essential nutrients shortage and increasing the overall immune resistance of the body.

According to the leading Russian nutritionists, recently there has been certain growth in the assortment of specialized food products. At the same time those food products are developed spontaneously, often without medical and biological justification of the product composition, without taking into consideration the directional properties and prophylactic purposes, without the availability of its proven properties. In addition, they note the necessity of the further development of the specialized food products assigned for dietary therapeutic nutrition in the Russian Federation [17].

Special approach is required to develop specialized meat-based food products for people with various non-infectious diseases, for whom food is one of the determining factors of health. Among food sources, meat is one of the most complicated and challenging matrices for creating dietary food products. The complexity of developing such products is caused by availability of a wide range of physiologically significant nutrients in meat on the one hand, and by availability of a range of anti-alimentary components (heme iron which serves as a possible reason of carcinogenicity, as well as saturated fats and cholesterol), on the other hand.

The strategies for healthy meat products development of reducing risk factors and enhancing the positive impact of meat on human health are being now actively studied around the globe via the approaches based on the inclusion of ingredients with proven therapeutic efficiency — prebiotics, dietary fiber, polyphenols, antioxidants, etc. It has been found that consumption of meat products fortified with dietary fiber prevents the diseases like coronary heart disease, diabetes, irritable bowel syndrome, obesity, etc. Dietary fiber, which has long story of being added to various minced meat products, is also associated with minimizing the carcinogenic effect of meat, reducing the period when feces remain in the colon and binding the minerals, thereby reducing heme activity. In addition, biologically active compounds such as polyphenols and antioxidants help prevent fat oxidation in meat [18].

[1] TR CU027/2012 Technical Regulations of the Customs Union "On safety of certain types of specialized food products, including dietary therapeutic and dietary preventive nutrition" (Adopted by the decision of the Council of the Eurasian Economic Commission of June 15, 2012 No. 34). Moscow, 2012. Retrieved from https://docs.cntd.ru/document/902352823. Accessed August 15, 2024 (In English)

Based on a number of scientific and practical works implemented by the authors of this article in the field of technologies of dietary therapeutic and prophylactic nutrition meat products, the prerequisites arose for systematization of requirements and using the principles of formulation of these food products. This contributed to the development of a unified methodology, which can be used as a comprehensive approach that combines various methods and integral indicators.

The aim of this work is formalization of the researches results related to the development of dietary therapeutic and prophylactic meat-based food products in the form of a methodology.

Objects and methods

The article describes the general algorithm for developing the specialized dietary therapeutic and prophylactic nutrition meat products, and demonstrates the implementation of its individual stages using the example of products for people with the most socially significant diseases like diabetes and cardiovascular disease.

The methodology algorithm is a sequence of the stages implemented during the food product development and a description of their content and practical implementation.

The research material was based on the modern approaches to the formulation of healthy food products for people with socially significant diseases, recommendations of the World Health Organization based on the analysis of scientific literature published in open and publicly available databases: ScienceDirect, eLIBRARY.RU, as well as on the results of our own research in the field of technologies for dietary therapeutic and prophylactic meat products.

As the objects of the research the authors used the specialized meat and meat-containing food products intended for therapeutic and prophylactic nutrition, developed by the authors: semi-finished beef products intended for people with diabetes; minced meat products for the correction of diets for people who suffer from cardiovascular diseases; products based on meat raw materials, for therapeutic (enteral) nutrition. Data on their ingredients composition, nutritional value, the results of their preclinical and clinical trials was used.

To select the criteria for the food composition parameters and to define their values, the main characteristics (properties) of specialized food products were formalized. Numerous parameters (nutritional value, content of critically important substances, criteria of nutrient adequacy) were analyzed. Nutrient adequacy was assessed with the software "System for designing and assessing the quality of multicomponent food compositions" by Lipatov's method [19].

Results and discussion

The sequence and content of the stages of meat and meat-containing food products development for dietary therapeutic and dietary prophylactic nutrition are presented below in the Figure 1.

Depending on the purpose, a specialized meat product can be either therapeutic or prophylactic. This property is determined taking into account the clinical condition of the consumer. The dietary therapeutic product is intended for nutritional support of people with nutritional deficiency developing against the disease background, used as the only source of nutrition. A dietary prophylactic food product is intended for its inclusion in the diet in order to prevent exacerbation of diseases and mobilize the body's immune defense.

Nutritional support is a therapeutic nutrition in the form of nutritional mixtures, the purpose of which is to provide the body with all the necessary nutrients using special nutritional methods that differ from the conventional food intake, which support is prescribed in various diseases. The enteral product based on meat can be used as a sole source of nutrition or used in combination with specialized enteral nutrition mixtures. It is assigned for consuming in accordance with the recommendation and under the supervision of a physician. The composition of the food product must fully satisfy the daily demand for essential nutrients, energy, vitamins, macro- and microelements. The volume of the product and the number of food intakes are determined by the physician depending on the age, body weight and clinical condition of the patient.

The formulation of specialized meat products for disease prophylactic nutrition is based on the application of traditional technologies of conventional meat food, modified as per the characteristics of the properties and chemical composition of meat and innovative ingredients that form the food matrix, their technological compatibility, and the assignment of the food product.

Special role belongs to medical and biological requirements (MBR), which have the most important role in the formation of the nutrient profile of a specialized meat product for both purposes: therapeutic and prophylactic. MBR are developed jointly by medical and technological process specialists and include requirements for the safety and physiological properties of the food product, taking into consideration the physiological and metabolic characteristics of the category of people whom this food is intended for. General MBR criteria for meat products have been established. The criteria are shown below in the Figure 2.

Medical and biological requirements determine the values and proportions of nutritional value parameters, calories content, functional ingredients content, as well as limitations on the content of critically important nutrients. Depending on the type of disease, the specialized food products may additionally contain protective food components or, in opposite, lack the nutrients that contribute to the disease progress. For example, diabetes and obesity require cutting down the consuming of easily digestible sugars in food; food recommended for cardiovascular pathology should contain less table salt, saturated fats and be rich in polyunsaturated fatty acids (PUFA), which

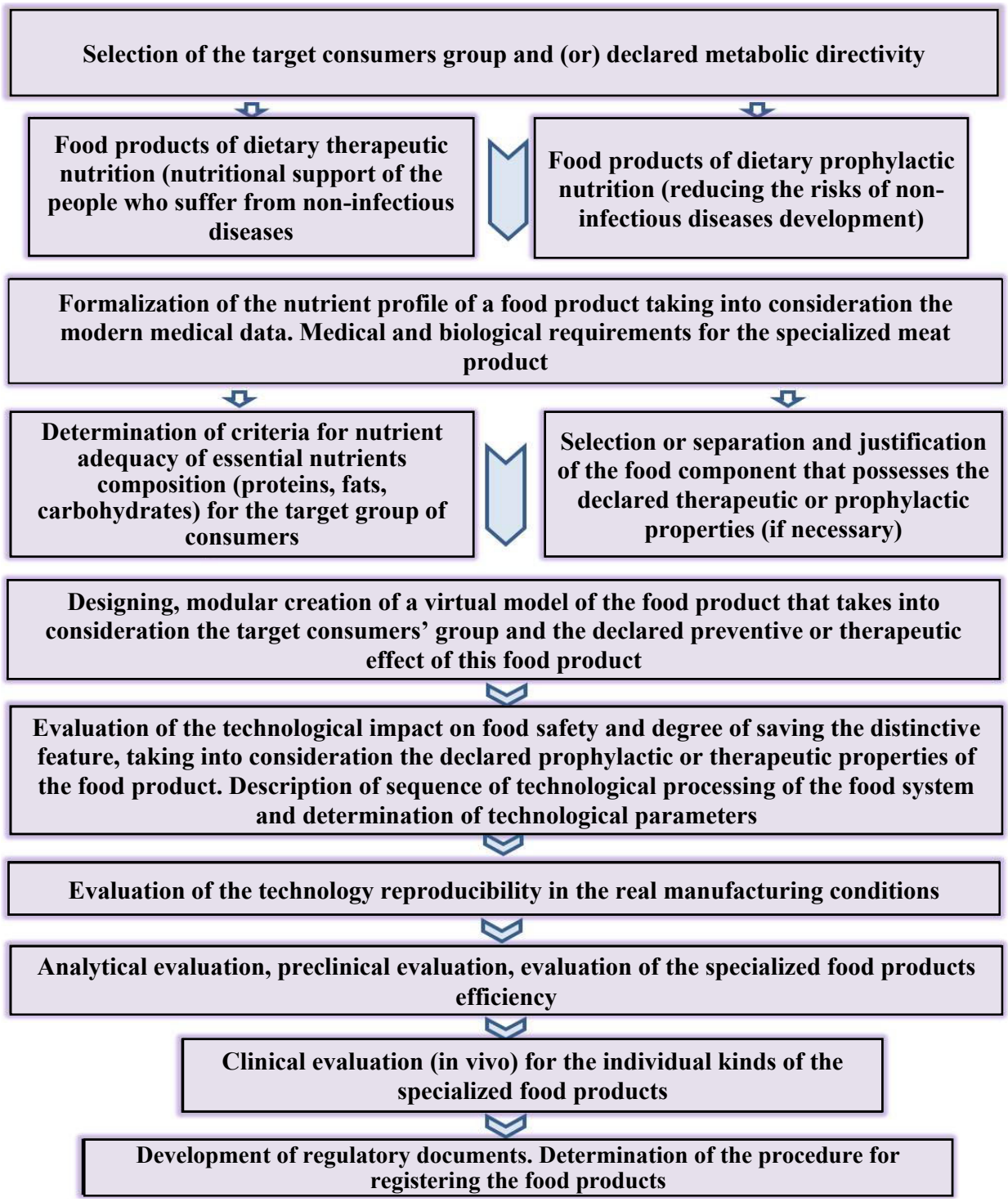

Figure 1. Scheme of specialized meat products development

provide an anti-inflammatory effect. Michalina Banaszak and colleagues presume the importance of not only the anti-inflammatory but also the modulating effect of polyunsaturated fatty acids EPA and DHA [20]. The available researches demonstrate that EPA and DHA supplements provide a beneficial effect on regulation of triglycerides, total cholesterol, insulin resistance, blood pressure, liver enzymes, inflammatory markers and oxidative stress. In addition, there are data of their potential benefit in terms of mitochondrial function, regulation of plasma lipoproteins and reduction in the risk of sudden cardiovascular events related to atherosclerotic plaque rupture [20].

To implement the above specified requirements, first of all it is necessary to select the relevant raw materials of animal and plant origin as the source of the main macronutrients (protein, fats and carbohydrates) that form the product matrix, then select the fortifying ingredients — the micronutrients and biologically active components with proven health benefits in case of certain diseases. Ingredients included into the composition of specialized meat products assigned for the patients with cardiovascular disease and with diabetes of the 2nd type shall be organoleptically and technologically compatible both with each other and with the product matrix, have no

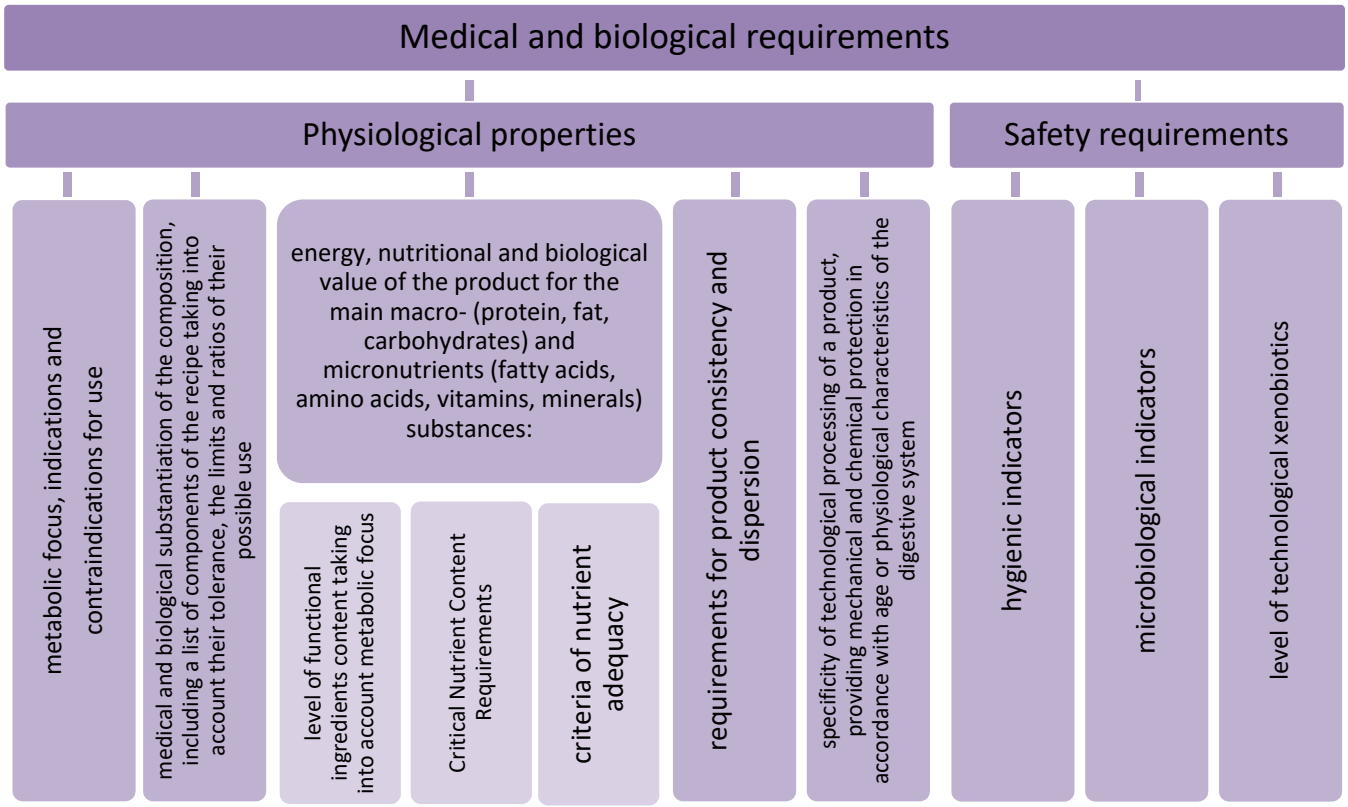

Figure 2. MBR structure and main criteria

hyperglycemic effect, help reduce cholesterol and regulate fat metabolism.

When forming the nutrient profile of products for both dietary therapeutic and dietary prophylactic nutrition, one of the main macronutrients is protein, which is involved into maintaining nitrogen balance, thus ensuring normal metabolism and preserving muscle tissue. Protein included in specialized food products should be complete, balanced in amino acids composition, and have high digestibility. When developing meat products, meat raw materials with high content of muscle tissue are selected as a basis, which ensures a high level of animal protein content in the finished food product. As an additional source of protein, it is possible to use milk protein (casein), or highly hydrolyzed milk protein, or whey protein, or vegetable protein to achieve optimal amino acid balance in reference to the standard of FAO/WHO [21].

Fat is also a significant macronutrient in food. Given the fact that meat fat has a specific fatty acid composition with saturated fats predominance, it is necessary to adjust the fat component of the product by creating a combination of fat obtained from meat raw materials and vegetable oils mixtures. The purpose of this correction is to ensure the required content of mono- and polyunsaturated fatty acids in the product, including the omega-3 family, which improve the lipid spectrum of the blood, help reduce the level of total cholesterol, provide the positive effect on the immune system and improve the inflammatory response.

To develop the high-quality composition of therapeutic enteral nutrition food, together with the need for protein and fat it is also necessary to provide a certain amount of non-protein calories due to carbohydrates for the adequate protein absorption by the body. For meat products of therapeutic nutrition, the carbohydrate profile is formed by inclusion into the recipe the slowly digestible and slowly absorbable carbohydrates, which do not increase the level of postprandial glycemia compared to the consumption of mono- and disaccharides.

Micronutrient deficiency is one of the risk factors for many diseases, including cardiovascular disease and diabetes. Taking this into account, it is recommended to include vitamins, minerals and other nutritionally significant ingredients, including those with antioxidant properties, into the composition of specialized food products in relevant quantities that ensure the correction of their insufficient consumption [22,23].

The content of each nutritionally significant component (biologically active substance) introduced into the food product must correspond to the daily norm and obey the following rule: in 100 g of the specialized prophylactic food products, or in a single portion of the formulated food product, the minimum content of the introduced nutritional component should be kept at the level of 15% from the recommended daily consumption of this component, and in case of a product for nutritional support the dose of introduced vitamins and minerals should cover 100% of the daily demand when consuming the established daily dose of this product.

When introducing a nutritionally significant component into food, it is necessary to ensure its safety, heat resistance and stability during storage. This fact is particularly important for the food products subjected to sterilization.

Moreover, for each nutrient-significant component the research methods with the sufficient detection limit must be known, which method is able to define the quantitative content of the introduced component to be detected even in small concentrations.

There is one more important condition for introduction of nutritionally significant components — this is the preservation of the nutritional value and consumer properties peculiar for this type of food product. The product based on meat proteins, intended for dietary therapeutic nutrition in the form of enteral nutrition, should be produced ready-to-consume and should be a sterilized homogenized mixture with a texture that allows it to be freely introduced through a gastrostomy tube ≥14 Fr (the outer diameter of the tube is 4.62 mm) via syringe as bolus or with the help of "natural gravity" (gravity). The food product should be a thick viscous-flowing homogenized mass made up of finely dispersed particles. For enteral nutrition products, the particle size is an important quality parameter, especially for the food products fed to the patient through probe tubes. The maximum possible decreasing of particles size of a meat product is necessary to increase the efficiency of gastrointestinal enzymes, which provide positive effect on the digestibility of the most important micro- and macronutrients. Increasing of dispersion degree is achieved by using technological methods aimed at reducing the particle size (high-pressure homogenization) in the technology of specialized meat products manufacture.

The appearance of the food product intended for dietary prophylactic nutrition should not differ from the relevant traditional food product, the color and taste peculiar to the product should correspond to the raw materials and the used components.

The next stage of the methodology implementation involves computer designing of a model recipe for the specialized meat product, taking into consideration the distinctive features of the meat raw material and the degree of nutritionally significant components introduction.

Depending on the species kind of the meat, and its anatomical and morphological origin, the meat may possess certain distinctive features that can be used to select the preferred meat raw material for the specific specialized food product, such as a source of the meat, or due to high content of protein, vitamins (B2, B9, B6, B12, PP), minerals (iron, zinc, selenium), low fat content, saturated fatty acids, as a source of omega-3 fatty acids.

To characterize the content and mutual balance of macro- and micronutrients and their components in the meat products of the specialized nutrition, the indicators of nutrient adequacy are used, like the amino acid composition of protein, fatty acid composition of the fat component, the content of metabolically dominant macro- and microelements, water- and fat-soluble vitamins. To assess the balance of fatty acids, fatty acid balance coefficients — R1…3 are used, taking into account the proportions of SFA, MUFA, PUFA sums in comparison to the similar criteria of the standard proportions. To assess the quality of protein in therapeutic nutrition products, the protein digestibility-corrected amino acid score (PDCAAS) is also used [24].

The criteria which the model recipe is assessed by are the nutritional value indicators and the values of the nutrient adequacy coefficients specified for specialized meat products, specified below in the Table 1. The adequacy indicators were defined empirically based on the results of numerous studies in the field of developing meat products for therapeutic and prophylactic nutrition purposes, taking into consideration the consumer characteristics and properties of the specialized meat products, the content of critically important nutrients, and the norms of physiological needs for energy and nutrients for the various groups of the population of the Russian Federation[2].

The above specified limit values are generalized, and for each specialized meat product it is necessary to establish individual quality and safety indicators, which should be included into the regulatory documentation for specific types and names of products.

Taking into consideration the range of recommendations for reducing the cardiology risk while developing minced meat products intended for the diet to prevent cardiovascular diseases, saturated fatty acids in the fat were partially replaced with polyunsaturated ones. Also, the level of table salt in the recipe was reduced by 50%, the vitamins with cardioprotective properties, vitamins and minor biologically active substances with profound antioxidant action that fight free radicals were added [25,26]. Modification of the fat composition allowed significant reducing the content of saturated fatty acids and increase the quota of unsaturated ones, ensuring a given PUFA/SFA ratio in the range of 1.6–2.3 and increase the total antioxidant capacity by 9 times in comparison with the similar traditional product. The introduction of an antioxidant complex contributed to the preservation of high antioxidant activity of the food product during its storage [27].

When developing a meat semi-finished food product for nutrition of the diabetic patients, it was taken into account that the main risk factor for carbohydrate metabolism disorders development is obesity. This requires cutting down the caloric content of the food product. In addition to carbohydrate metabolism disorders, the increased content of fats in the blood plays a major role in the progression of *diabetes mellitus*. Because of that it is necessary to significantly cut down the level of saturated animal fats in the diet, yet ensuring the intake of monounsaturated and polyunsaturated fatty acids (the oils with omega-3, omega-6, phospholipids). Another important factor is the inclusion of dietary fiber and hypoglycemic substances into the food product. The plants like amaranth, green beans, oregano,

[2] Methodological recommendations MP 2.3.1.0253-21 "Norms of physiological needs for energy and nutrients for various groups of the population of the Russian Federation". Moscow: Garant, 2021. Retrieved from https://www.garant.ru/products/ipo/prime/doc/402716140. Accessed July 29, 2024. (In Russian)

Table 1. Limit values of nutritional value indicators, coefficients adequacy of amino acid and fatty acid compositions of products, content of critically important substances

Indicator	Nutrient content and amino acid and fatty acid balance indicators	
	Meat-based product for dietary therapeutic nutrition	Meat product for dietary prophylactic nutrition
Protein, not less than	15% of calories	10 g/100 g of product or 12% of calories
Fat, no more	35% of calories	12 g/100 g of product
Table salt, g/100 g	—	no more than 1.8
ΣNFA	no more than 20 g/100 g of lipids	no more than 10% of calories
ΣMUFA	50–60 g/100 g of lipids	not less than 10% of calories
ΣPUFA	27–31 g/100 g of lipids	6–10% of calories
Minimum score, fractional unit (C_{min})	0.85–1.0	
Utility score, fractional units (σ)	0.85–1.0	
Comparable excess score, g/100 g protein (U), no more than	7	10
Protein digestibility corrected amino acid score (PDCAAS)	1	—
Fatty acid balance score, fractional units R_{Li}, I=1…3	0.85–1.0	
ω-6/ω-3 fatty acid ratio	3–4,4:1	3–5:1

chicory, garlic, celery, as well as vegetables that contain no starch, feature the effect similar to insulin.

The next stage related to the evaluation of the technological impact on the food product safety, consists of establishing the impact of technological processes on the formation of "thermal" carcinogens (melaidins, peroxides, heterocyclic amines, acrylamide, etc.), which is especially peculiar for the meat products exposed to high-temperature processing sterilization). The influence of temperature-time factors on the indicators and biological value of the food product, organoleptic parameters and the safety of the introduced fortifying components is also evaluated.

It is necessary to note that the type of heat treatment plays a key role in influencing the oxidation processes direction. If the development of meat products for specialized nutrition requires changing the fat composition of the product by introducing PUFA sources, it is necessary to focus on researches of the heat treatment effect on their preservation. PUFAs are most susceptible to the high temperatures exposure as they form the various radicals, which subsequently are able to form malonic dialdehyde [28]. Fats oxidation is accompanied by deterioration of their organoleptic properties and the formation of various oxidation products — first peroxides, and then polymeric compounds with a toxic action. The oxidation resistance of the fat component of the food products with various types of vegetable oils is evaluated by changes in acid and peroxide numbers. It is also known that some methods of thermal processing of high-protein foods of animal origin produce the potentially carcinogenic compounds, including heterocyclic aromatic amines — chemical compounds that have in their structure at least one aromatic ring and one amino group [29].

One of the purposes of the methodology stage is to cutting down to minimum the risks related to the formation in specialized meat products of chemical compounds xenogeneic to the human body. This can be achieved by refusing from the aggressive methods of technological processing (high temperature, smoking, etc.), by standardizing the content of xenobiotics in the finished food product, by refusing from the process food additives or significantly reducing their content, and by using modern control methods that guarantee food safety.

At this stage, pilot production of the full-scale food product samples is run in order to analytically evaluate their compliance with the model samples and to study the effect of introduced biologically active components on the organoleptic properties of the finished food product. The stage involves the development of technological modes for the preparation of biologically active substances for their further introduction, selection of the stage and effective method of their introduction, and the analysis of consumer properties of the developed product. If the full-scale samples is relevant to the model sample in terms of its quality, safety, and organoleptic characteristics, they proceed to the next stage which consists of assessment of possibility to reproduce the developed technology in the real conditions of an operating enterprise, taking into consideration its raw material sources and technological base.

An important stage of the specialized meat products development algorithm is a comprehensive evaluation of efficiency and safety, including three areas: analytical assessment, evaluation *in vitro, in vivo, ex vivo experiments,* and clinical tests.

The food product is analytically evaluated for its compliance with the quality and safety indicators specified at the initial stage of development of the specialized food product, including the actual content of the introduced nutritionally significant components and biologically active substances. Safety standards are formed depending on the type of meat products, and are adopted in accordance with

the requirements of technical regulations TR CU021/2011[3], TR CU034/20134, TR CU027/2012[5].

Preclinical efficiency of specialized food products consists of studying biological properties (antioxidant, immunomodulatory, cytoprotective, hypolipidemic, hypoglycemic, neuroprotective, etc.) and identifying the specific activity of functional ingredients introduced into the food product in *in vitro, in vivo,* and *ex vivo* experiments.

To define the therapeutic and prophylactic nutrition effects of the food product in *in vivo* experiments on the laboratory animals, the methods of experimental modeling of human diseases are used. Experimental modeling of functional states on animals is carried out by varying diet components, introducing chemical agents — damage inducers, surgical intervention. The main markers that indicate the condition of animals are the parameters of blood serum, urine, liver-aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), low-density lipoprotein cholesterol (LDL–C), high-density lipoprotein cholesterol (HDL–C), triglycerides TG, histological studies of tissues, etc.

Preclinical evaluation of the specialized meat product assigned for the patients with cardiovascular diseases was conducted on sexually mature female laboratory rats of the NISAG line, which rats were obtained at the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences from a population of Wistar rats by selection for hypertensive increase response when exposed to mild emotional stress [30]. The rats of this line represented a model of hypertensive disease similar to that in humans. Aging rats showed a well-pronounced increase in blood pressure, which makes them a worthy model for research. The results of noninvasive measurement of blood pressure using a tail cuff, immunoenzyme indices associated with damage to the heart muscle, vasoconstriction and increased blood pressure, and histological studies of rat heart samples were used as markers of their health condition [31].

To model *diabetes mellitus* in laboratory animals, a drug was administered into their bodies that caused the development of disease symptoms: glucosuria, hyperglycemia, polydipsia, polyphagia and polyuria, hematocrit increase, decrease in blood clotting time and an increase in its viscosity, activation of cytolytic processes in liver and pancreas of the animals. To assess the health condition of the animals, blood parameters were studied, including changes in blood glucose levels, the content of ketones and glucose in the urine, and activity of AST and ALT enzymes [32].

When positive results are obtained in animals, the desired procedure of confirmation the prophylactic dietary meat products efficiency is their evaluation in clinical conditions.

The clinical trials are mandatory for dietary therapeutic food products.

The purpose of clinical studies is to evaluate in a clinical setting the effect of the food product on the patients' clinical status and biochemical parameters, including parameters of carbohydrate and lipid metabolism in the blood serum of the patients with type 2 *diabetes mellitus*, their medical examination, anthropometric measurements, physical and instrumental evaluation of the functional state of their internal organs and body systems, biochemical studies of blood serum, general blood test and urine test, the level of daily glucosuria, hypolipidemic effect (level of total cholesterol, triglycerides, VLDL–C, LDL–C and the value of the atherogenicity coefficient) [33].

During clinical trials of enteral therapeutic nutrition products, during the patient's stay in the clinic, the tolerance is assessed using a specially designed chart, where patients every day mark the dynamics of their complaints and disease symptoms severity (dryness, bitterness in the mouth, heaviness in the stomach after intake of the food product, presence and severity of abdominal pain, flatulence, heartburn, nausea, feeling of heaviness after eating, incomplete bowel movement), an evaluation of the stool parameters dynamics. The results of laboratory tests and evaluation of the quality of life, body composition indicators are compared before and after the relevant modification of the diet [34].

The final stage of the food product development is its state registration, which is a mandatory procedure of compliance confirmation for the specialized food products.

Conclusion

The principles of developing the specialized food products differ from the traditional technologies, and that circumstance requires the comprehensive approach to their creation, taking into consideration the specified properties, purpose and type of the food product. For meat products the comprehensive approach provides for taking into consideration many factors, including the characteristics of the properties and chemical composition of meat and traditional technological methods of the ready food products manufacturing.

Using the theoretical and experimental approaches, a methodology for creating specialized meat products for dietary nutrition has been developed, which methodology exercises a comprehensive approach starting from the development of the food product till its manufacturing. The developed methodology can be used as a tool for scientifically justified development of specialized meat products and justification of their efficiency.

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SENSORY TESTING AND QUALITY MAINTENANCE OF HAMBURGERS CONTAINING SOYBEAN MEAT

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Keywords: *consumer preference, food quality, meat substitute, soya protein, color and pH, viable bacteria count, peroxide content*

Abstract
In the past few years, lifestyle-related diseases have been increasing, and meat intake has been linked to this increase. Therefore, in recent years, there have been attempts to use soybeans as a substitute for meat. Soybeans exhibit antioxidant and antimicrobial effects. Therefore, processing foods using soybeans may preserve food quality because the main factors causing food deterioration are oxidation and microbial growth. To verify the quality-retention effect of soybeans, we conducted a sensory test on hamburgers, the quality of which readily deteriorates over time. We investigated the percentage of soybean meat that would be acceptable in a hamburger and quality retention with the addition of different amounts of soybean meat. We found that hamburgers with soybean meat were accepted by more than half of the participants when the soybean meat content was ≤50%. In terms of changes over time in quality-related factors, the L- and b-values (lightness/brightness and yellowness/blueness, respectively) were higher, a-value (redness/greenness) was lower, and pH was higher in hamburgers that contained soybean meat than in those without added soybean meat. Furthermore, an increase in the bacteria count and peroxide content was suppressed in hamburgers containing soybean meat. The results indicate that hamburgers containing soybean meat are of higher quality than those made with 100% animal meat. The use of soybeans as a meat substitute in hamburgers can help mitigate the rise of lifestyle-related diseases linked to high meat consumption.

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Introduction

Over the last few years, the number of cases of lifestyle-related diseases, such as obesity, cancer, and diabetes, has increased rapidly [1], becoming a social problem worldwide. According to the World Health Organization and 2012 World Health Statistics, lifestyle-related diseases accounted for 63% of global deaths or 36 million deaths in 2008, with a higher proportion of middle-aged and elderly people. By 2030, the number of deaths from lifestyle-related diseases is projected to increase to 55 million, including 13 million from cancer [2]. In addition, in the 2023 WHO report, the total number of global deaths from NCDs (non-communicable diseases, which include lifestyle-related diseases plus chronic obstructive pulmonary disease and mental health) is projected to reach 90 million by 2048 [3]. In 2024, the WHO reports that while many health risks due to smoking and poor sanitation have decreased in recent years, health risks associated with activities such as alcohol consumption and hypertension are very high, and the obesity epidemic shows no signs of recovery [4].

The increase in cancer incidence is closely related to dietary changes, and a strong association between increased animal protein intake and cancer risk has been reported [5]. In men, the relative risk of colon and colorectal cancer considerably increases with high meat intake [5], and the overall risk of developing colorectal cancer increased in those consuming red meat and processed meat [6]. Furthermore, the incidence of colorectal cancer has been reported to be higher in Hawaii and Los Angeles than in Miyazaki, Japan, where meat consumption is high [7].

In recent years, soybean, a vegetable with high protein content, has been attracting attention as an alternative to animal protein sources. Soybeans have approximately 0.41and 2.9 times higher fat and protein content, respectively, than beef [8], offering the advantage of efficient protein intake. Soybeans can be consumed as boiled beans, but they are often processed into various foods, with many being sold as processed products. Processed soybean-based products include natto (fermented soybeans), okara (bean curd), soymilk, and soybean meat. Among them, tofu and

okara are widely consumed as processed soybean products, and they can be mixed with ground meat to prepare hamburgers with reduced animal protein content.

Okara tempeh (OT), a soybean product, has been shown to have high water retention, oil absorption, and antioxidant capacities, making it an excellent processed food material [9]. A previous study measured the peroxide content in cupcakes with and without OT stored at 40 °C. The absorbance value at 550 nm reflecting the peroxide content of cupcakes without the additive increased from 0.30 at week 0 to 1.0 at week 3, whereas that of cupcakes with OT increased from 0.30 at week 0 to 0.75 at week 3, indicating that OT inhibits fat and oil oxidation [9]. Given that ground meat has a high lipid content, food processing using soy products is expected to prevent lipid oxidation owing to the antioxidant effects of soy. Furthermore, a study investigated the minimum inhibitory concentration of ethanol extracts of different soybean powders for various bacteria. The inhibition zones of *Escherichia coli* were 3.16 and 9.33 mm upon treatment with extracts of black and yellow soybeans, respectively, whereas those of *Bacillus subtilis* were 7.16 and 10.0 mm, respectively [10], showing that the ethanol extract of yellow soybeans exerts a stronger antibacterial effect than that of black soybeans. In addition, when the antibacterial effects of soybean-derived saponin and chloramphenicol, an antibiotic, were measured on *E. coli*, the bacterial inhibition zone was 13.2 and 12.0 mm, respectively [11]. This finding demonstrated that for *E. coli*, the antibacterial effect of saponin extracted from soybeans is stronger than that of chloramphenicol and confirmed the antimicrobial property of soybeans. However, it has been reported that tofu and okara have a disadvantage as hamburger additives because hamburgers with these soybean products are whiter and smoother than those without them, resulting in the loss of the unique texture of meat [12]. Among processed soybean products, soybean meat, which has meat-like color and texture, can be added to hamburgers without changing their color or texture. Soybean meat is made of dried, processed soy protein [13] and can be used in a variety of dishes by tempering with water. The consumption of soybean meat in Japan has considerably increased from 23,560 tons in 2010 to 33,297 tons in 2019 [14], indicating a growing demand.

Soybeans contain several bioactive components, such as soy saponins, soy proteins, lecithin, and soy isoflavones; the latter, in particular, has many health-promoting effects [15]. Furthermore, as isoflavones are a type of polyphenol with antioxidant and antimicrobial effects [16], the production of hamburgers using soybean meat may preserve their quality without sacrificing flavor or texture.

Generally, food quality is evaluated based on physical, chemical, and biological factors, as well as sensory factors determined over time. However, the parameters that substantially affect food quality vary depending on the food type. Hamburgers are made by kneading, preparing, and baking ground meat. Although hamburgers are cooked, ground meat has a larger surface area exposed to air and is more likely to lose quality than steaks and grilled meat [17]. Parameters related to changes in meat quality include pH, oxidation, and microorganism count. Previous studies have reported that in raw beef slices, the pH increased from 5.91 on day 1 to 7.14 on day 18 [18], whereas in uncooked hamburger meat, it increased from 5.51 on day 1 to 7.11 on day 7 [19], demonstrating a faster pH increase in minced meat. It has also been reported that the disruption of muscle cell membranes during meat mincing accelerates the interaction between unsaturated fatty acids and oxidative substances, accelerating lipid oxidation and acidification [19]. Additionally, the content of 2-thiobarbituric acid reactive substances in beef steak reportedly increased from 0.130 mgMDA/kg on day 1 to 3.41 mgMDA/kg on day 16, whereas that in ground beef increased from 2.02 mgMDA/kg on day 1 to 6.91 mgMDA/kg on day 16 [17], indicating that lipid oxidation proceeds at a faster rate in ground beef than in steak meat. Furthermore, the microbial load in raw beef slices was found to be 3.60 logCFU (colony-forming unit)/g on day 1 and 9.50 logCFU/g on day 18 [18], whereas that in ground beef was found to be approximately 3.00 logCFU/g on day 1 and 10.0 logCFU/g on day 12 [20], showing a faster increase in the latter.

In the present study, the meat parameters that affect quality considerably, namely, color, pH, microbial count, and lipid oxidation [18], were analyzed. The effect of soybean meat on quality retention in the production of hamburgers was examined. In addition, a questionnaire survey was conducted to determine the percentage of soybean meat that is acceptable in hamburgers. Moreover, the quality change in hamburgers achieved with the acceptable soybean meat-to-meat ratio was investigated, and the quality retention of soybean meat was verified. The findings of the present study could facilitate a reduction in the intake of animal proteins, which, in turn, could decrease the risk of development of lifestyle-related diseases, while maintaining the overall quality of hamburgers by using soybeans. Furthermore, the results of this research could contribute to the reduction of food loss and human health, in addition to contributing to the popularization of soybeans globally.

Objects and methods

Hamburger and soybean meat preparation

A mixture of ground beef (thigh; domestic, Australia, and USA) and pork (shoulder; domestic, USA, and Canada) (7:3 ratio) purchased as an off-the-shelf product at a retail store in Hachioji City, Japan, was used to prepare hamburger patties. Dried soybean meat (100 g; Nichie Corporation, Nagoya, Japan) was placed in a frying pan with approximately 500 mL of water and brought to a boil to produce soybean meat. Subsequently, the soybean meat was allowed to simmer over low heat for 5 min and drained. For the sensory test, approximately 1,000 g of ground meat was prepared, and hamburger patties containing 0%, 30%,

50%, 60%, and 70% soybean meat were made. The hamburgers were grilled at 190 °C until browned on both sides, and then steamed at 170 °C for 5 min with the lid on. For measuring the changes in quality, hamburger patties containing 0%, 30%, and 50% soybean meat were prepared, grilled at 190 °C until browned on both sides, and then steamed at 170 °C for 5 min with the lid on. The hamburgers were then placed on a tray, covered with Saran Wrap, and stored at 5 °C for 21 days.

Sensory test

Fifteen hamburger patties (three patties per group) were prepared for the sensory test. In this test, a score of 4 (very good), 3 (good), 2 (bad), or 1 (very bad) was assigned for the appearance, texture, firmness, and juiciness of the hamburger, as described by Shibata-Ishiwatari et al. [12]. A score was assigned for each parameter, and the patty was given an overall score. The percentage of respondents who thought the hamburger was acceptable was determined. The sensory test was conducted three times with 10 (male: 7; female: 3) participants aged between 21 and 22 years. The study was approved by the Ethics Committee of the Tokyo University of Technology (No. E22BS-011).

Quality assessment

For the assessment of the quality of hamburgers, 18 hamburger patties containing 0%, 30%, and 50% soybean meat (six hamburger patties per group weighing approximately 30 g each) were prepared and evaluated, as more than 50% of the sensory test respondents found them acceptable.

Color and pH

A colorimeter (Shenzhen ThreeNH Technology Co., Ltd., Guangzhou, China) and pH meter (Sato Shoji Co., Ltd., Kanagawa, Japan) were used to measure the surface characteristics and pH, respectively, on three different locations on the surface of the hamburgers 4, 7, 12, 18, and 21 days after they were cooked. The L- (lightness/brightness), a- (redness/greenness), and b- (yellowness/blueness) values were measured.

Viable bacteria count

Hamburger samples (2 g) were added to 20 mL of 0.9% sterile saline solution. Once the meat sank to the bottom, the supernatant was collected and diluted. Subsequently, 100 µL of the diluted supernatant was smeared on a standard agar medium using a bacteria spreader (SFC–1000; AS ONE CORPORATION, Osaka, Japan). The culture medium was then incubated at 35 °C for 48 h, and the bacteria count was determined on days 4, 7, 12, 18, and 21.

Peroxide content

Hamburger samples (5 g) were placed in a triangular flask and mixed with 30 mL of isooctane (Kanto Chemical Co., Ltd., Tokyo, Japan) and acetic acid (FUJIFILM Wako Pure Chemical Co., Ltd., Osaka, Japan) at a 2:3 ratio. Subsequently, a saturated solution of 0.01 mM potassium iodide (FUJIFILM Wako Pure Chemical Co., Ltd.) was added, and the mixture was shaken for 1 min and placed in the dark at 25 °C for 5 min. Thereafter, 30 mL of pure water was added, and the solution was shaken for 5 min, after which 0.5 mL of 1% starch solution (FUJIFILM Wako Pure Chemical Co., Ltd.) was added. The peroxide content was determined on days 4, 7, 12, 18, and 21 through titration using the following formula:

$$\textit{Peroxide content} = 10 \times (V - v) \times F/C;$$

where V is the titration volume; v is the blank; F is 1.0; and C is the sample volume (g).

Statistical analysis

The Wilcoxon signed rank test and *t*-test were used to determine differences in the means between the groups. The Wilcoxon signed rank test was performed using the R software (The R Foundation, Vienna, Austria); the *t*-test and analysis of variance were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Results with p-value < 0.05 were considered statistically significant.

Results and discussion

Sensory test

Table 1 shows the results of the questionnaire survey on the sensory quality of hamburger patties with different percentages of soybean meat. Eighty percent of the respondents found the hamburgers were acceptable when the percentage of soybean meat was 0%–50%, whereas <40% found them acceptable when the percentage of soybean meat was 60%–70%. The appearance scores tended to decrease with an increase in the amount of soybean meat ($p > 0.05$). In terms of texture, the score of 34–31 for hamburgers containing 30%–50% soybean meat, respectively, was higher than the score of 24 for

Table 1. Sensory test results for hamburgers with different percentages of soybean meat

Soybean meat percentage (%)	Appearance score	Texture score	Hardness score	Juiciness score	Acceptable percentage (%)
0	35	30	30	34	100
30	34	34	32	31	90
50	34	31	32	26	80
60	31	28	30	23	40
70	27	24	26	21	30

hamburgers with 70% soybean meat ($p < 0.05$). The firmness score of 32 for hamburgers with 30% soybean meat was higher than the score of 26 for hamburgers with 70% soybean meat ($p < 0.05$). Additionally, the juiciness score of 34–31 for hamburgers containing 0%–30% soybean meat, respectively, was higher than the score of 23–21 for hamburgers containing 60%–70% soybean meat ($p < 0.05$), respectively.

Regarding the appearance of hamburgers, the evaluation scores tended to increase as the percentage of soybean meat in the hamburger patties decreased (Table 1). When the meat surface temperature reaches approximately 150 °C, a reaction (Maillard reaction) occurs between amino compounds, such as proteins, and carbonyl compounds of reducing sugars; this reaction causes a significant change in meat color from red/pink to grayish brown during the heating process [19]. The beef and pork used in this study each contained 26 g of amino acids, and 0.4 and 0.1 g of sugar, respectively, per 100 g (edible portions), whereas the soybeans contained 1.8 mg of amino acids and no sugar [20]. Therefore, as the amount of added soybean meat increased, the sugar content in hamburgers decreased, which could have inhibited the Maillard reaction, resulting in decreased appearance evaluation scores. Furthermore, the red color of raw meat is due to myoglobin, which changes to grayish-brown when heated, and then to pink during cooling [19]. Soybean meat does not contain myoglobin; thus, this color change does not occur, which may explain the lower appearance score of hamburgers with soybean meat than that of hamburgers without it.

In terms of hamburger texture and firmness, the evaluation scores tended to decrease as the percentage of soybean meat in the hamburgers increased. In a previous study, when minced OT was added to standard hamburgers (700 g minced ground beef, 5 g onion, 5 g breadcrumbs, 5 g salt, and 0.1 g nutmeg), the scores for texture and firmness increased with increasing OT quantity [5]. A difference in preference for meat firmness has been suggested based on gender, with female students reportedly regarding "softness" as a more important quality of beef [20]. Moreover, in a study by Shibata-Ishiwatari et al. [12], all participants were female individuals. In the present study, we enrolled seven male and three female individuals; the decreasing scores for texture and firmness with increasing soybean meat content in hamburgers may be explained by the small proportion of female individuals enrolled in this study.

In a previous study, hamburgers were prepared with yams and okara or tofu as substitutes for chicken eggs, breadcrumbs, and milk as the binder. The yams and okara were rated as "non-cohesive" and "dry," with significantly decreased juiciness compared to that of 100% animal meat hamburgers [12]. In the present study, the juiciness evaluation scores also decreased with increasing soybean meat content in hamburgers.

In a study by Matsuo [9], hamburgers containing up to 20% OT were acceptable. When hamburgers contained over 30% OT, the total score decreased. In the present study, we observed a similar trend, with decreasing overall evaluation scores for hamburgers with increasing soybean meat content.

Quality assessment

Color and pH

Figure 1 shows changes in the L-values of hamburgers containing various percentages of soybean meat when refrigerated for 21 days. For hamburgers containing 0% soybean meat, the L-values varied between 35.8 and 42.8 throughout this period, revealing no increasing or decreasing trend ($p > 0.05$). The L-values for hamburgers with 30% soybean meat varied between 36.8 and 44.0 on days 1–18 ($p > 0.05$) and slightly increased to 50.8 on day 21 ($p > 0.05$). Hamburgers with 50% soybean meat showed L-values varying between 39.0 and 45.7 on days 1–7 ($p > 0.05$) and slightly increasing to 51.2 on day 21 ($p > 0.05$). Additionally, a trend toward increasing L-values with increasing amount of soybean meat in hamburgers was observed.

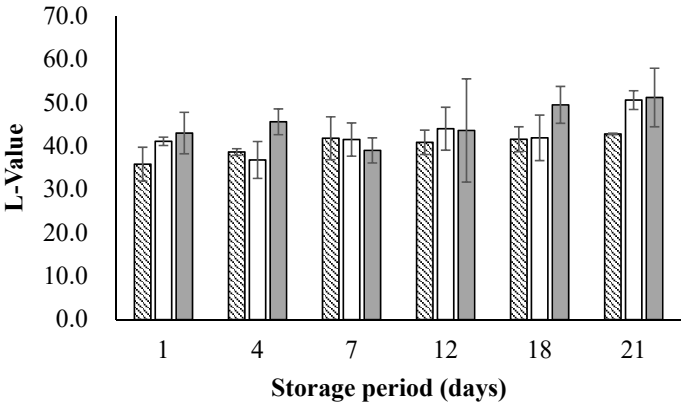

Figure 1. L–Values of hamburgers with different percentages of soybean meat during 21 days of refrigerated storage. Shaded, white, and gray bars represent hamburgers with 0%, 30%, and 50% soybean meat, respectively. Data are presented as mean ± standard deviation (n = 5)

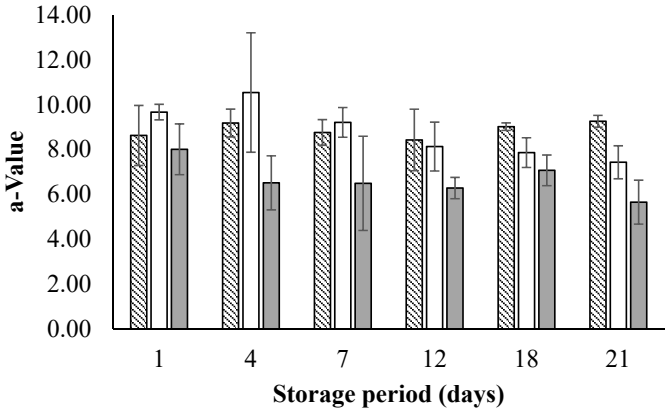

Figure 2. a-Values of hamburgers containing different percentages of soybean meat during 21 days of refrigerated storage. Shaded, white, and gray bars represent hamburgers with 0%, 30%, and 50% soybean meat, respectively. Data are presented as mean ± standard deviation (n = 5)

Figure 2 shows changes in the a-values of hamburgers containing various percentages of soybean meat when refrigerated for 21 days. For hamburgers containing 0% soybean meat, the a-value varied between 8.44 and 9.27 over the 21-day period, showing no increasing or decreasing trend ($p > 0.05$). For hamburgers with 30% soybean meat, the a-value was 9.68 on day 1, increasing slightly to 10.6 on day 4 ($p > 0.05$) and decreasing slightly to 7.44 on day 21 ($p > 0.05$). The a-values of hamburgers containing 50% soybean meat ranged from 5.66 to 8.02 on days 1–21, revealing no increasing or decreasing trend ($p > 0.05$). Additionally, a trend toward decreasing a-values with increasing amount of soybean meat in hamburgers was observed.

Figure 3 shows changes in the b-values of hamburgers with various percentages of soybean meat when refrigerated for 21 days. For hamburgers with 0% soybean meat, the b-value was 11.7 on day 1, increasing slightly to 19.9 on day 4 ($p > 0.05$) and varying between 18.5 and 19.9 on days 4 to 21; there was no increasing or decreasing trend ($p > 0.05$). For hamburgers with 30% soybean meat, the b-value range was 18.4–22.2 over the 21-day period, with no increasing or decreasing trend ($p > 0.05$). The b-values for hamburgers with 50% soybean meat varied between 16.8 and 20.8 over the 21 days, without an increasing or decreasing trend ($p > 0.05$).

Figure 4 shows changes in the pH of hamburgers containing different percentages of soybean meat when refrigerated for 21 days. Hamburgers with 0% soybean meat had a pH of 5.94 on day 1, increasing to 6.22 on day 12 ($p < 0.05$), decreasing to 5.95 on day 18 ($p < 0.05$), and increasing again to 6.12 on day 21 ($p < 0.05$). For hamburgers with 30% soybean meat, the pH was 6.26 on day 1, increasing slightly to 6.46 on day 12 ($p > 0.05$), and decreasing slightly to 6.36 on day 21 ($p > 0.05$). In hamburgers containing 50% soybean meat, the pH was 6.22 on day 1 and increased to 6.70 on day 21 ($p < 0.05$). The pH of hamburgers tended to increase as the amount of soybean meat increased.

Figures 1–3 show that when hamburgers without soybean meat were stored at 5 °C for 21 days, there was no trend of increase or decrease in the L-, a-, and b-values. For lamb cooked on the grill at 200 °C and refrigerated at 2 °C for 14 days, the L-, a-, and b-values reportedly vary between 50.5 and 51.2, 7.9 and 8.0, and 13.8 and 15.3, respectively, from day 1 to day 14 with no increasing or decreasing trend [21]. In the hamburger without soybean meat in this study, the L-, a-, and b-values fluctuated between 35.8 and 42.8, 8.62 and 9.27, and 15.0 and 18.5, respectively, over a 21-day period with no increasing or decreasing trend, and the same trend was observed, indicating that no color change occurs over time during storage. However, compared with those for lamb, the a and b values in this study were close, whereas the L value in this study was low. The L value, which indicates brightness, decreases as the aminocarbonyl reaction proceeds with heating in the case of direct grilling [22], and in this study, the hamburger was grilled at a heating temperature of 180 °C until the hamburger became charred. On the contrary, lamb was cooked on the grill at 200 °C. However, the internal temperature of the meat was about 72 °C because the grill cooking method heats the meat as a whole without concentrating the heat in one place. Therefore, the surface temperature was lower on the grill compared with that of direct heat, which may have resulted in lower L-values in this study.

Figures 1–3 show that the L-values increased and a-values decreased as the percentage of soybean meat added in the hamburger increased from 0% to 50%; the L-values reportedly decrease due to the aminocarbonyl reaction [22]. A comparison of the composition of meat and soybeans, per 100 g of edible portion, revealed that both beef and pork contained 0.4 and 0.1 g of sugar, respectively, and 26 g of amino acids, whereas soybeans contained 1.8 mg of

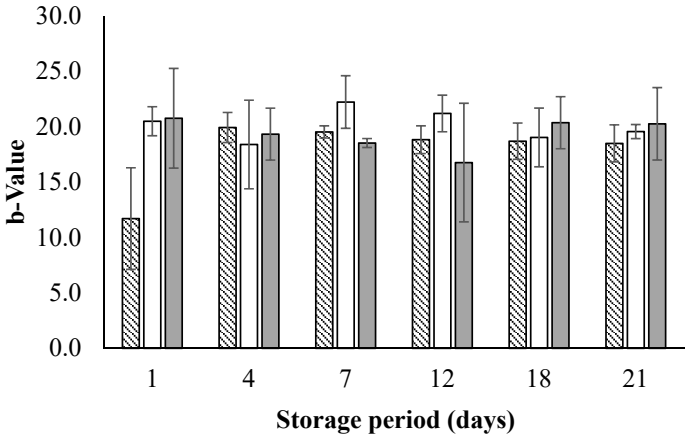

Figure 3. b-Values of hamburgers with different percentages of soybean meat during refrigerated storage for 21 days. Shaded, white, and gray bars represent hamburgers with 0%, 30%, and 50% soybean meat, respectively. Data are presented as mean ± standard deviation (n = 5)

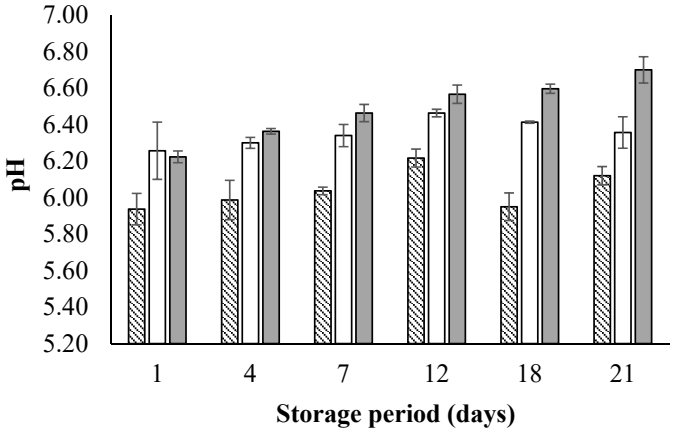

Figure 4. pH of hamburgers with various percentages of soybean meat during 21 days of refrigerated storage. Shaded, white, and gray bars represent hamburgers with 0%, 30%, and 50% soybean meat, respectively. Data are presented as mean ± standard deviation (n = 5)

amino acids and no sugar [8]; both amino acid and sugar contents in soybeans were lower than those in meat. Therefore, the addition of soybean meat is not a good option. We believe that the addition of more soybean meat increased the L-value because the Maillard reaction did not occur due to the lower amount of sugar. In addition, the color of the meat became lighter with the addition of soybeans, which may have resulted in a tendency for the a-value to decrease with increased soybean addition.

Figure 4 shows that the pH of all the hamburgers examined tended to increase with time when stored at 5 °C for 21 days. The pH also increased as the percentage of soybean meat increased. The pH of ground beef stored at 4 °C for 12 days reportedly varies between 5.68 and 6.16 [23], and that of ground pork stored at 4 °C for 14 days varies between 5.88 and 6.00 [24]. In the present study, the pH of the hamburger with 0% soybean meat refrigerated for 21 days ranged from 5.94 to 6.22 from day 1 to day 21, respectively.

Figure 4 shows that as the amount of soybean meat in the hamburger increased, the pH also increased; the pH of soybeans (kouji-irasu, ayamidori, green and black soybeans) is in the range of 6.29–6.62, whereas the pH of cooked ground meat is 6.16 [23]. The pH of the soybeans reflected the pH of the hamburgers with soybeans; therefore, it can be considered that the pH increased as the percentage of soybean meat increased.

Viable bacteria count

Figure 5 shows changes in the viable bacteria count in hamburgers containing different percentages of soybean meat and refrigerated for 21 days. In hamburgers with 0% soybean meat, the bacteria count was 5.83 logCFU g^{-1} on day 1, increasing to 11.9 logCFU g^{-1} on day 21 ($p < 0.05$). In hamburgers with 30% soybean meat, this count was 6.30 logCFU g^{-1} on day 1 and 11.8 logCFU g^{-1} on day 21 ($p < 0.05$). In hamburgers with 50% soybean meat, the bacteria count was 6.05 logCFU g^{-1} on day 1, increasing to 6.54 logCFU g^{-1} on day 4 ($p < 0.05$), decreasing to 5.43 logCFU g^{-1} on day 7 ($p < 0.05$), and increasing again to 9.99 logCFU g^{-1} on day 21 ($p < 0.05$). Moreover, the bacteria counts increased over time. After day 4, the bacteria counts were lower in hamburgers with 50% soybean meat than in those with 0% and 30% soybean meat.

In UV-irradiated raw beef, the microbial count was reportedly around 3.70 logCFU g^{-1} on day 1, increasing to approximately 9.50 logCFU g^{-1} on day 18 [18], which is consistent with the results of the present study. Additionally, in our study, the bacteria counts in hamburgers with 30% and 50% soybean meat were lower than those in hamburgers without soybean meat. It has been reported that soybeans contain the isoflavones 2′-hydroxyerythrin A and isoerythrinin A, which have antimicrobial properties [25]. The presence of these isoflavones could be a reason for the decreasing bacteria counts with increasing soybean meat content in hamburgers observed in our study.

Peroxide content

Figure 6 shows changes in the peroxide content in hamburgers containing different percentages of soybean meat during refrigerated storage for 21 days. In hamburgers with 0% soybean meat, the peroxide content was 10.1 meq/kg^{-1} on day 1, increasing to 18.7 meq/kg^{-1} on day 12 ($p < 0.05$), and decreasing to 12.8 meq/kg^{-1} on day 21 ($p < 0.05$). In hamburgers with 30% soybean meat, the peroxide content was 7.10 meq/kg^{-1} on day 1, decreasing to 4.07 meq/kg^{-1} on day 12 ($p < 0.05$), and increasing to 6.21 meq/kg^{-1} on day 21 ($p < 0.05$). In hamburgers with 50% soybean meat, this value ranged between 2.84 and 4.89 meq/kg^{-1} on days 1–21, with no increasing or decreasing trend ($p > 0.05$). Additionally, the peroxide content decreased with increasing soybean meat content in hamburgers.

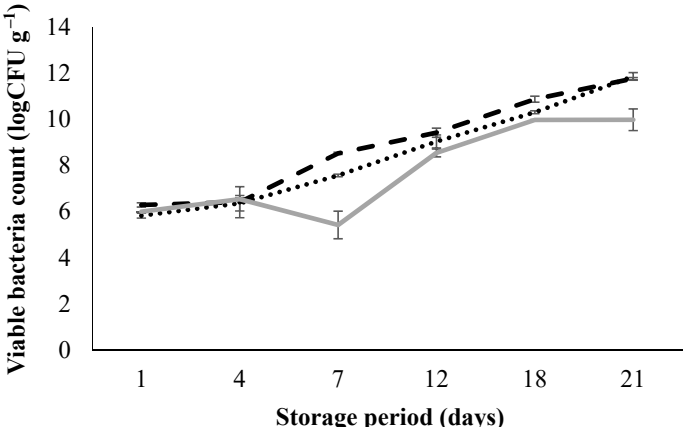

Figure 5. Viable bacteria counts in hamburgers with different percentages of soybean meat during refrigerated storage for 21 days. Dotted, dashed, and gray lines represent hamburgers with 0%, 30%, and 50% soybean meat, respectively. Data are presented as mean ± standard deviation (n = 5)

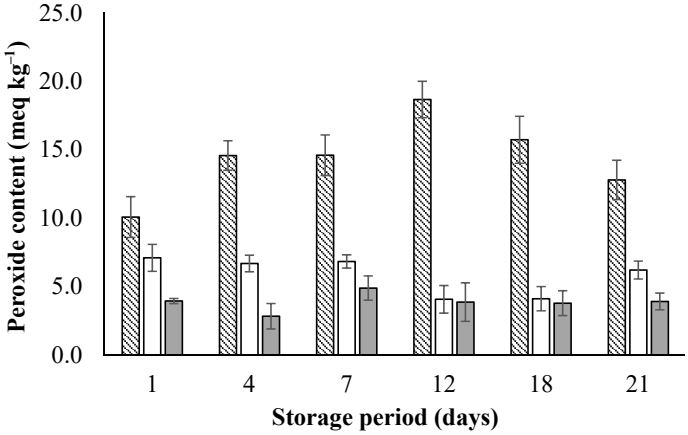

Figure 6. Peroxide content in hamburgers with different percentages of soybean meat during 21 days of refrigerated storage. Shaded, white, and gray bars represent hamburgers with 0%, 30%, and 50% soybean meat, respectively. Data are presented as mean ± standard deviation (n = 5)

In our study, the peroxide content increased slowly over time. Additionally, this parameter tended to increase and then decrease over time in hamburgers containing soybean meat. In a previous study on dry fermented sausages, the addition of soy flakes decreased the peroxide content to below 4.0 meq/kg^{-1} [26]. It has also been reported that OT suppressed fat and oil oxidation in ground hamburger meat and cupcakes [12]. Therefore, adding soybean meat to hamburgers may have reduced the peroxide content and suppressed its increase over time. Moreover, in the present study, the peroxide content in hamburgers without soybean meat reached the maximum on day 12 and then decreased. Ghimire et al. [27] reported that the peroxide content in beef meatballs was 0.25 meq/kg^{-1} on day 1, reached its maximum of 1.27 meq/kg^{-1} on day 6, and then decreased. This change happens because the degradation of unstable hydroperoxides leads to the formation of aldehydes, ketones, and epoxides, causing the accumulation of secondary oxidation products; thus, it is only reliable to measure the peroxide content during the early stages of lipid oxidation, when the peroxide content increases to its maximum value, as it decreases with extended storage time [27]. Therefore, malondialdehyde, reflecting the peroxide content, is useful for evaluating food oxidation in the early stages [28]. In this study, the peroxide content increased and then decreased with storage time in hamburgers without soybean meat, but these changes were not observed in hamburgers with soybean meat, suggesting that the progress of oxidation was delayed in hamburgers with soybean meat. The hamburger containing soybean meat was considered to show delayed progression of oxidation.

Soybeans contain various antioxidants, and one of the fat-soluble antioxidants is tocopherol. Tocopherols, which are present in soybean oil, reportedly have antioxidant properties, and prevent fat and oil oxidation by scavenging free radicals generated in the initial stages of oxidation, indicating that oxidation is suppressed by soybean oil [29]. A previous study measured the peroxide content of soybean and sesame oils and reported that it increases from 1.2 meq/kg^{-1} at month 0 to 21.6 meq/kg^{-1} at month 44 and from 0.2 meq/kg^{-1} at month 0 to 109.8 meq/kg^{-1} at month 44, respectively. This finding indicated that oxidation was suppressed in soybean oil [30].

Soybean meat is composed of dried, processed soy protein, and its characteristics vary depending on the type of soybean used. Currently, there are only a few soybean meat products available; therefore, comparing various types of soybean meat products is challenging. Novel soybean meat products are expected to be introduced in the market. Moreover, meat quality may depend on its region of origin; however, this was not examined in this study, warranting further investigation to understand the effects of different regions of origin of meat on its quality. As soybean meat has only recently been commercialized and there are only a few studies on this product, the present study is a pioneer in the characterization of soybean meat and investigation of its possible benefits. Nevertheless, further characterization of soybean meat should be conducted in the future.

Conclusion

This study investigated the percentage of soybean meat acceptable in a hamburger and quality preservation in hamburgers produced with added soybean meat. In the sensory evaluation of hamburgers with soybean meat, more than 80% of the respondents answered that hamburgers were acceptable when the soybean meat content was ≤50%, whereas less than 40% answered that hamburgers were acceptable when the soybean meat content was ≥60%. Regarding quality deterioration over time, specifically color, hamburgers containing soybean meat tended to have higher L- and b-values and lower a-values than hamburgers without added soybean meat. Hamburgers containing soybean meat tended to have a higher pH and lower bacteria count than hamburgers without soybean meat. Furthermore, hamburgers containing soybean meat had lower peroxide content than pure animal protein hamburgers. Therefore, hamburgers containing less than 50% soybean meat are deemed acceptable and their quality preservation is higher than that of 100% animal protein hamburgers.

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