



The effect of organic acids mixture in the form of glycerids on the regulation of intestinal barrier function in piglets using SCFA-M: analysis of molecular markers

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AG: Conceptualization; Formal analysis; Investigation; Writing — original draft preparation.
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The functions of the intestinal system are crucial for animal health and productivity, as they ensure digestion, nutrient absorption, immune homeostasis, and microbiota regulation. Molecular markers of the intestinal system, such as occludin (OCL), fibronectin (FN), interferons (IFN- α , IFN- γ), and caspase-3 (Casp-3), serve as sensitive indicators of its functional state. This article presents the results of an experimental study on the effects of short-chain fatty acids (SCFA-M, C3-C12) on the expression of molecular markers of intestinal barrier function in three-way crossbred piglets. A total of 100 piglets aged 42 days were selected for the study and divided into control and experimental groups. The diet of the experimental group was supplemented with SCFA-M. Expression levels of molecular markers were assessed in the duodenum using the Western blot method. Obtained results showed a significant increase in the expression of OCL, FN, and IFN- α in the experimental group, indicating improved barrier function, extracellular matrix stability, and immune response. Specifically, by day 56, FN expression increased by 46.80 % ($P < 0.001$), OCL by 16.78 % ($P < 0.001$), and IFN- α by 20.06 % ($P < 0.05$) compared to the control group. Contrary, decreased levels of IFN- γ and Casp-3 indicated reduced inflammation and apoptotic activity. The correlations between molecular marker expression and metabolic parameters were analysed to clarify the interaction of these indices. Notably, in 56-day-old piglets of the control group, OCL expression negatively correlated with blood total protein levels ($r = -0.83$; $P < 0.05$). In 77-day-old animals of the experimental group, correlations were found between FN and OCL expression and serum calcium levels ($r = -0.90$; $P < 0.05$). These findings demonstrated the positive effects of SCFA-M on immune regulation, proinflammatory balance, and the maintenance of intestinal barrier integrity in piglets. The presented data may be applied in veterinary practice for the prevention of intestinal infections, enhancement of pathogen resistance, and optimization of animal productivity.

Key words: intestinal system, molecular markers, SCFA-M, piglets, intestinal barrier function, occludin (OCL), fibronectin (FN), interferons (IFN- α , IFN- γ), caspase-3 (Casp-3)



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Introduction

The intestinal system functions are critically important for nutrient absorption, immune defense, and microbiota regulation [8, 21]. It ensures the processes of digestion and absorption of essential nutrients, maintains a barrier against pathogens, supports microbiota balance, and regulates metabolic and immune homeostasis [8]. Enhancements or disruptions of these functions can significantly affect overall health and growth, particularly in animals such as piglets, for whom intestinal development is crucial for productivity and disease resistance [4]. Currently, there is a growing need for a better understanding of the physiological functions of the intestinal system and the factors that influence its homeostasis.

Molecular markers of the intestinal system serve as powerful tools for assessing the functional status of the gut, particularly its barrier function. Since protection against enteropathogens is ensured by a complex interplay of cell-to-cell junctions and cell-extracellular matrix adhesions, the level and state of adhesion proteins can serve as sensitive indicators of intestinal barrier integrity and density [16]. For example, proteins such as OCL, claudins, and zonulin-1 play a key role in forming tight junctions that prevent the penetration of pathogens and toxins into the submucosa [3]. Changes in the expression or structure of these proteins can signal barrier dysfunction, which often accompanies inflammatory processes or infectious diseases [4]. At the same time, FN, which connects cells to the extracellular matrix, contributes to tissue stability and damage repair [5]. Monitoring molecular markers not only allows for the diagnosis of functional disorders but also provides a means to evaluate the effectiveness of therapeutic and preventive interventions aimed at maintaining or restoring intestinal health. This opens broad prospects for the application of molecular markers in veterinary practice, particularly in enhancing animal resistance to intestinal infections and supporting their productivity.

The aim of study was to find out the effect of SCFA-M blend on the gut barrier integrity and immunity in the thin intestine of the piglets as a chain of studies conducted for other species [12].

Materials and Methods

The experiment was conducted using 100 three-way crossbred *DanBred* piglets aged 42 days, which were divided into two groups: control and experimental (50 animals per group). Piglets were housed in group pens in compliance with EU stocking-density standards: 0.20 m²/pig for the 10–20 kg body-weight range and 0.30 m²/pig for 20–30 kg; the actual allowances provided were 0.22–0.24 and 0.33–0.36 m²/pig, respectively. Water was available *ad libitum*; complete compound feed was offered *ad libitum* according to identical feeding regimens in both groups (except for the experimental additive in

the treatment group). The environmental conditions were maintained within stable limits: temperature 22–26 °C, relative humidity 55–65 %, mechanical ventilation; a fixed photoperiod of 16L:8D was observed. Prior to the trial, all animals underwent clinical examination; only clinically healthy piglets were enrolled. Weaning from the sows occurred on day 26 of life; the trial began at 42 days of age. From 42 to 77 days of age, the treatment group received in the diet a composition of monoglycerides of short- and medium-chain fatty acids (SCFA-M, C3–C12), custom-manufactured for PARTNERAGRO2016 LLC at the production facilities of NETAG B.V. (Netherlands), at a dose of 1.0 kg per metric tonne of feed. According to the certificate of analysis, the proportions of glyceride fractions (w/w %) were: monoglycerides 31 %, diglycerides 18 %, triglycerides 1 %, free glycerol 14 %. The acids esterified with glycerol included propionic (C3), butyric (C4), caprylic (C8), capric (C10), and lauric (C12) acids. Inclusion was performed in a production mixer with process control of batch homogeneity.

To assess the expression of molecular markers related to the intestinal barrier function, five piglets from each group were euthanized at 42, 56, and 77 days of age. The samples of the duodenum were selected from every animal and washed with phosphate saline buffer (PBS). The tissue samples were immediately frozen and stored at –22 °C no longer than 2 weeks before start of analysis.

Duodenal tissue samples were homogenized with using RIPA buffer to extract both soluble and insoluble protein fraction. The extraction was carried out 50 minutes at 4 °C and homogenates were centrifuged 45 minutes at 20,000 g. Western blot analysis carried out as it reported early [7]. Protein extracts from the intestinal tissue samples were separated by electrophoresis in polyacrylamide gels (PAAG) with an acrylamide gradient concentration of T = 7–18 % [11]. Protein bands were transferred from the gel to nitrocellulose membranes with an electric field (current of 150 mA) during 60 minutes. The blocking of unspecific adsorption on nitrocellulose membrane surface was carried out using bovine serum albumin (BSA) 1 % solution in PBS. Following blocking, membranes were incubated with a 1:1500 dilution of specific primary antibody in PBS contained 1 % dry milk. All primary antibody were purchased from *Santa Cruz Biotechnology* including anti-caspase-3, anti-fibronectin, anti-occludin, anti-IFN- γ , and IFN- α (respectively sc-56046; sc-271098; sc-133256; sc-390800; sc-373757). After washing membrane was probed with secondary HRP conjugated antibody at 60 min then washed and developed on the X-ray film with advanced ECL method. The results of total protein content were expressed as percentages relative to the control group. The total protein concentration in each sample was determined by the Bradford assay to equalize the obtained data with WB method [2].

Metabolic status was assessed using serum biochemical parameters. At 42, 56, and 77 days of age, whole blood without anticoagulant was collected from 10 piglets per group to obtain serum. Serum concentrations of total

protein, albumin, globulins, total calcium, and inorganic phosphorus were determined on a *Miura-200* automated analyzer (Italy) using commercial kits from *Spinreact* (Spain). The protein coefficient (albumin-to-globulin ratio) and the Ca/P ratio were calculated. Associations between biochemical parameters and the expression of molecular markers in the piglet duodenum were evaluated by Pearson's correlation analysis.

Statistical analysis was performed using specialized software *Prism 10*. The descriptive statistics used in this study included: M — mean; SD — standard deviation. Differences were considered statistically significant at $P < 0.05$ (including $P < 0.01$ and $P < 0.001$).

Results and Discussion

The results of present study have established strong correlations between the modulation of molecular markers in the duodenum of piglets at different stages of the experiment ($r = 0.82\text{--}0.99$; $P < 0.05\text{--}0.01$). This may indicate complex regulation of immune response, barrier functions, and apoptosis, which are altered under the influence of the experimental conditions, particularly the supplementation of SCFA-M.

A significant impact of SCFA-M supplementation was observed in respect with the expression level of molecular markers in the duodenum of piglets (fig.). Notably, in 56-day-old piglets from the experimental group, expression levels increased significantly: IFN- α up to 138.14 % (SD = 17.69 %), FN up to 163.06 % (SD = 1.63 %), and OCL up to 123.42 % (SD = 5.99 %). These levels were respectively 20.06 % ($P < 0.05$), 46.80 % ($P < 0.001$), and 16.78 % ($P < 0.001$) higher compared to the control group. At the same time, IFN- γ expression reached only 85.97 % (SD = 8.70 %) and Casp-3 dropped to 64.44 % (SD = 8.19 %), which were 57.12 % ($P < 0.001$) and 54.76 % ($P < 0.001$) lower, respectively, than in the control group.

These data reflect significant changes that may be attributed to various mechanisms of SCFA-M action. The increase in IFN- α expression indicates activation of the immune response in the duodenum of the experimental piglets [6]. IFN- α is known for its antiviral activity, suggesting improved intestinal barrier resistance to pathogens [18]. Its elevated level points to the stimulatory effect of SCFA-M on the immune system.

The marked increase in FN expression is an indicator of improved extracellular matrix (ECM) structure and rehabilitation [10]. FN is a ECM component involved in tissue repair and the maintenance of intestinal wall structural integrity [19]. Its upregulation reflects the beneficial impact of SCFA-M on tissue homeostasis.

The increased expression of OCL suggests enhanced epithelial barrier function [1]. OCL is a vital component of tight junctions, which prevent the penetration of pathogens and toxins [20]. Its higher concentration indicates stabilization of barrier integrity under the influence of SCFA-M.

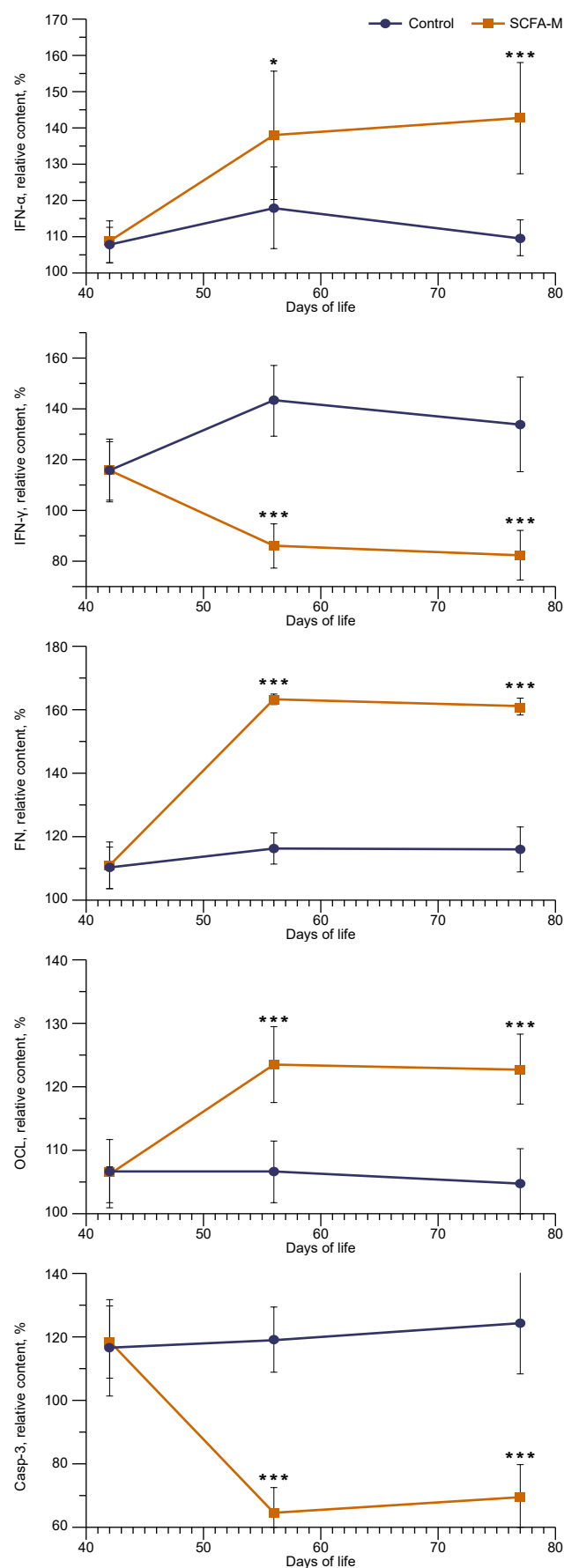


Fig. Content of molecular markers in the duodenum of piglets under the influence of SCFA-M, % ($M \pm SD$; $n=5$).
Note. * — $P < 0.05$; ** — $P < 0.01$; *** — $P < 0.001$ compared to the control group.

The intestinal mucosa is constantly exposed to the luminal environment, including bacteria, toxins, and pathogens, making it highly susceptible to inflammation. Both exogenous and endogenous factors can disrupt the balance of the gut microbiota, physiological processes, and immune functions of the mucosa, leading to reduced feed intake, diarrhea, and impaired growth [14]. The epithelial cells of the mucosal lining in the gastrointestinal tract constitute the largest immune organ in pigs. The health of these cells is essential for pig growth and development, as they are responsible for secretion and absorption [17]. Molecular markers such as FN, OCL, Casp-3, IFN- α , and others play critical roles in gut health, contributing to the barrier function, immune defense, and structural stability of the gastrointestinal tract [13, 18].

The observed differences in molecular marker expression in the duodenum of piglets under the influence of SCFA-M persisted until the final day of surveillance (day 77). Specifically, by day 77 in the experimental group, the expression of IFN- α was 33.10 % higher ($P < 0.001$), FN increased by 44.95 % ($P < 0.001$), and OCL rose by 17.95 % ($P < 0.001$), whereas the levels of IFN- γ and Casp-3 decreased by 51.47 % ($P < 0.001$) and 54.68 % ($P < 0.001$), respectively, compared to the control group. The expression of IFN- γ is indicative of inflammatory activity, as IFN- γ is a key cytokine promoting inflammation [15]. Therefore, its reduction may suggest an anti-inflammatory effect of SCFA-M, helping to reduce the risk of developing intestinal inflammatory diseases. Meanwhile, Casp-3 is a known marker of apoptosis [22], and its decreased expression indicates reduced apoptotic activity in the intestinal epithelial cells [23]. This reduction may result from the stabilizing influence of SCFA-M on cellular metabolism and stress mitigation.

The findings confirmed the positive effects of SCFA-M on immune response regulation and the maintenance of intestinal barrier integrity in piglets. The increased expression of IFN- α , FN, and OCL emphasizes enhanced resistance of the intestinal epithelium and improvement in its structural condition. At the same time, the reduced expression of IFN- γ and Casp-3 indicates diminished inflammation and apoptosis, reflecting the stabilizing and protective effects of SCFA-M.

It is important to note that in 56-day-old piglets, the modulation of molecular marker expression was closely associated with the protein metabolism changes, whereas in 77-day-old animals, these associations were more strongly related to trace element metabolism. This may reflect age-related changes in metabolic processes and differential effects of SCFA-M on these age groups.

In 56-day-old piglets of the control group, the expression of OCL in the duodenum showed a negative correlation with total protein ($r = -0.83$; $P < 0.05$) and albumin ($r = -0.81$; $P < 0.05$) levels in blood serum (table). This suggests that reduced OCL expression may be linked to elevated serum protein components. These changes

could reflect adaptive processes of the intestinal barrier in response to increased protein metabolism typical for this age group. A positive correlation was found between Casp-3 levels and the serum protein coefficient ($r = 0.82$; $P < 0.05$) in piglets of the experimental group, indicating that increased apoptosis (Casp-3) may be associated with intensified protein metabolism, potentially reflecting active regeneration or enhanced cellular turnover under the influence of SCFA-M.

On the 77-day-old piglets the control group, a positive correlation was observed between IFN- γ and Casp-3 levels and the calcium-to-phosphorus ratio in serum ($r = 0.81$ – 0.92 ; $P < 0.05$ – 0.001). This suggests that changes in the expression of inflammatory and apoptotic markers may be associated with mineral balance, reflecting age-related shifts in metabolism. Casp-3 expression showed a negative correlation with phosphorus levels ($r = -0.81$; $P < 0.05$), indicating a possible reduction in apoptotic activity under phosphorus deficiency or altered phosphorus metabolism.

The effect of SCFA-M application was accompanied by the inverse correlations between FN and OCL expression in the duodenum and serum calcium levels ($r = -0.90$ to -0.85 ; $P < 0.05$) in the group of 77-day-old piglets. This may suggest that elevated mineral levels in the blood could be associated with reduced expression of these markers, potentially indicating alterations in the structural integrity of the intestinal barrier. Casp-3 expression in the duodenum also showed a negative correlation with phosphorus levels ($r = -0.83$; $P < 0.05$), which may point to the influence of SCFA-M in reducing apoptotic activity under conditions of altered mineral balance.

The inclusion of SCFA-M in the piglets' diet positively affects the expression of key molecular markers of intestinal barrier function, such as OCL, FN, and IFN- α , indicating improvements in the integrity and functional condition of the epithelial barrier. The decreased levels of IFN- γ and Casp-3 in the experimental group suggest an anti-inflammatory effect of SCFA-M and a reduction in epithelial cell apoptotic activity, which contributes to tissue stabilization and a lowered risk of developing inflammatory intestinal diseases.

The established correlations between the expression of molecular markers and metabolic indicators (serum protein and mineral levels) reflect the integrated interaction of SCFA-M with protein and mineral metabolism in piglets. The effects of SCFA-M vary with the age of the animals: in 56-day-old piglets, its influence was more pronounced on protein metabolism, while in 77-day-olds it primarily affected mineral metabolism.

The results indicate that SCFA-M supplementation in piglet feeding may serve as an effective strategy to enhance resistance to intestinal infections, maintain productivity, and support intestinal health. These findings highlight the potential for incorporating SCFA-M into veterinary practice to improve intestinal system function and increase the efficiency of piglet rearing.

Table. Associations between blood biochemical parameters and the expression of molecular markers in the piglet duodenum (r)

Indicators	Control group					Experimental group				
	IFN- α	IFN- γ	FN	OCL	Casp-3	IFN- α	IFN- γ	FN	OCL	Casp-3
42 days										
Total protein	-0.19	-0.32	-0.50	-0.61	-0.15	-0.54	-0.44	-0.49	-0.65	-0.49
Albumin	0.70	0.67	0.47	0.43	0.76	0.77	0.58	0.65	0.71	0.62
Globulin	-0.31	-0.44	-0.58	-0.70	-0.28	-0.71	-0.56	-0.63	-0.77	-0.61
A/G	0.47	0.54	0.69	0.78	0.38	0.82	0.71	0.73	0.82	0.74
Ca	-0.39	-0.53	-0.50	-0.48	-0.48	-0.04	-0.18	-0.03	0.14	-0.12
P	0.08	0.36	0.30	0.39	0.29	-0.52	-0.66	-0.48	-0.43	-0.64
Ca/P	-0.34	-0.53	-0.49	-0.49	-0.47	0.34	0.33	0.32	0.43	0.37
56 days										
Total protein	-0.63	-0.50	-0.59	-0.83	-0.45	0.12	0.26	0.20	0.13	0.21
Albumin	-0.54	-0.39	-0.52	-0.81	-0.36	0.04	0.19	0.14	0.05	0.17
Globulin	-0.38	-0.38	-0.32	-0.31	-0.31	0.17	0.28	0.22	0.19	0.21
A/G	-0.22	-0.09	-0.23	-0.49	-0.10	-0.09	-0.02	-0.02	-0.12	0.01
Ca	0.06	-0.27	-0.30	-0.07	-0.28	-0.53	-0.42	-0.55	-0.62	-0.56
P	0.33	0.52	0.51	0.21	0.60	-0.06	0.04	0.14	0.11	0.25
Ca/P	-0.01	-0.32	-0.34	-0.07	-0.34	-0.34	-0.31	-0.49	-0.51	-0.55
77 days										
Total protein	-0.57	-0.55	-0.48	-0.49	-0.50	-0.62	-0.55	-0.41	-0.52	-0.72
Albumin	-0.59	-0.61	-0.61	-0.57	-0.59	-0.03	0.02	0.09	-0.10	0.10
Globulin	0.34	0.40	0.46	0.38	0.41	-0.47	-0.45	-0.38	-0.34	-0.63
A/G	-0.48	-0.54	-0.56	-0.50	-0.54	0.36	0.37	0.32	0.23	0.53
Ca	0.54	0.65	0.51	0.59	0.70	-0.78	-0.79	-0.90	-0.85	-0.69
P	-0.66	-0.70	-0.62	-0.68	-0.81	-0.77	-0.69	-0.74	-0.80	-0.83
Ca/P	0.73	0.81	0.68	0.77	0.92	0.30	0.19	0.13	0.26	0.46

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Вплив суміші органічних кислот та гліцеридів на регуляцію бар'єрної функції кишечника поросят за використання SCFA-M: аналіз молекулярних маркерів

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Функції інтестинальної системи мають вирішальне значення для здоров'я та продуктивності тварин, оскільки забезпечують травлення, засвоєння поживних речовин, підтримку імунного гомеостазу та регуляцію мікробіоти. Молекулярні маркери кишкової системи, як-от оклюдин (OCL), фібронектин (FN), інтерферони (IFN- α , IFN- γ) та каспаза-3 (Casp-3), є чутливими індикаторами її функціонального стану. У статті представлені результати експериментального дослідження впливу коротколанцюгових жирних кислот (SCFA-M, C3–C12) на експресію молекулярних маркерів бар'єрної функції інтестинальної системи у поросят трьохпородного гібриду генетики *DanBred*. Для дослідження було відібрано 100 поросят віком 42 дні, які розділили на контрольну та дослідну групи. До раціону поросят дослідної групи додавали SCFA-M. Експресію молекулярних маркерів визначали у дванадцятипалій кишці методом *Western blot*. Результати демонструють значне підвищення експресії OCL, FN та IFN- α у дослідній групі поросят, що свідчить про покращення бар'єрної функції, стабільності екстрацелюлярного матриксу та імунної відповіді. Зокрема, на 56-й день експресія FN зросла на 46,80 % ($P < 0,001$), OCL — на 16,78 % ($P < 0,001$), а IFN- α — на 20,06 % ($P < 0,05$), порівняно з контрольною групою. Водночас зниження рівня IFN- γ та Casp-3 вказує на зменшення запальних процесів та апоптотичної активності. У статті розглянуто кореляційні зв'язки між експресією молекулярних маркерів та метаболічними параметрами. Зокрема, у 56-добових поросят контрольної групи експресія OCL демонструвала негативну кореляцію з рівнем загального білка в крові ($r = -0,83$; $P < 0,05$). У 77-добових тварин дослідної групи встановлено зв'язки між експресією FN та OCL з рівнем кальцію в сироватці крові ($r = -0,90$; $P < 0,05$). Результати дослідження свідчать про позитивний вплив SCFA-M на регуляцію імунної відповіді, зменшення запалення та підтримку цілісності кишкового бар'єру у поросят. Представлені дані можуть бути використані у ветеринарній практиці для профілактики кишкових інфекцій, підвищення стійкості тварин до патогенів та оптимізації їх продуктивності.

Ключові слова: інтестинальна система, молекулярні маркери, SCFA-M, поросята, бар'єрна функція кишечника, оклюдин (OCL), фібронектин (FN), інтерферони (IFN- α , IFN- γ), каспаза-3 (Casp-3)