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Katarzyna MICHAŁEK, Agnieszka KOLASA-WOŁOSIUK[1], Łukasz STAŚKIEWICZ[2]

RENAL EXPRESSION OF AQUAPORIN 2 (AQP2) OF GROWING PIGLETS FED DIET SUPPLEMENTED WITH INULIN AND PROBIOTICS

EKSPRESJA AKWAPORYNY 2 (AQP2) W NERKACH PROSIĄT ŻYWIONYCH PASZĄ Z DODATKIEM INULINY I PROBIOTYKÓW

Department of Physiology, Cytobiology and Proteomics, West Pomeranian University of Technology Szczecin, Poland
[1]Department of Histology and Embryology, Pomeranian Medical University, Szczecin, Poland
[2]The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences Jabłonna, Poland

Streszczenie. Stosowanie pasz z dodatkiem probiotyków i inuliny korzystnie wpływa na mikroflorę jelit. Tak wzbogacona dieta i tym samym utrzymanie pożądanej flory bakteryjnej w jelitach ma wielokierunkowe, prozdrowotne działanie dla zwierząt. Jednym z nich jest zwiększona biodostępność i wchłanianie mikro- i makroelementów. Wzrost przyswajalności w jelitach, a następnie wzrost koncentracji w osoczu krwi wielu pierwiastków powinien sprowokować homeostatyczną odpowiedź nerek w zakresie regulacji wydalania wody. W związku z powyższym postanowiliśmy przetestować hipotezę badawczą, w której zakładamy, że w nerkach świń karmionych paszą z dodatkiem probiotyków i inuliny wzrośnie ekspresja akwaporyny 2 (AQP2) – białka, które odgrywa istotną rolę w nerkowym zatrzymywaniu wody. Badania przeprowadzono na 16 samcach mieszańcach rasy Danbred x Duroc. Na podstawie analizy IHC stwierdzono, że u zwierząt z dietą zmodyfikowaną ekspresja AQP2 wzrasta głównie w błonie szczytowej komórek głównych kanalików zbiorczych. Za pomocą techniki Western blot wykazano, że wzrasta również ekspresja ogólnej ilości AQP2 w rdzeniu nerek. Obserwowane zmiany lokalizacji i ekspresji AQP2 wskazują na zwiększone nerkowe wchłanianie wody. Wzrost resorpcji wody najprawdopodobniej w odpowiedzi na dodatni bilans wielu komponentów wydaje się wspierać korzystny efekt podaży probiotyków i inuliny w diecie.

Key words: pigs, renal function, aquaporin 2, diet, inulin, probiotic.
Słowa kluczowe: prosięta, czynność nerek, akwaporyna 2, dieta, inulina, probiotyk.

INTRODUCTION

The introduction by the European Union a total ban on antibiotic growth promoters in animal feedstuffs caused that researchers began to look for other additives that could replace the previously used antibiotics. Amongst numerous biological feed components, probiotics and inulin proved to have outstanding health-promoting properties. Probiotics, which are also referred to as functional food, are in fact selected strains of bacteria and yeast, which are complementary to the natural intestinal microbiota. Inulin, on the other hand, which represents the so-called non-digestible food ingredients, promotes the growth and

Corresponding author – Adres do korespondencji: Katarzyna Michałek, Department of Physiology, Cytobiology and Proteomics, West Pomeranian University of Technology, Szczecin, Doktora Judyma 6, 71-466 Szczecin, Poland, e-mail: katarzyna.michalek@zut.edu.pl

activity of beneficial gut microflora and inhibits the growth of pathogenic microorganisms (Apolinário et al. 2014; Sari et al. 2014). Feeding such enriched diet allows maintaining desired intestinal microbiota and, in consequence, has multidirectional, beneficial, health-promoting effects. So modified diet, both in animals and humans, stimulates the immune system, regulates the lipid profile, reduces the level of blood plasma cholesterol, and increases the bioavailability of minerals (Zduńczyk 2004; Salah et al. 2013). In addition, it has been shown that probiotics and inulin in the diet lowers the level of metabolites, has anticarcinogenic effect, supports the treatment of diabetes and increases the production of group B vitamins (Meyer and Stasse-Wolthuis 2009; Bezirtoglou and Stavropoulou 2011).

Inulin- and/or probiotics supplemented diet fed to animals positively affects their fitness and health status, reduces feed intake, increases body weight gains and, as a result, improves the efficiency of animal production. In humans, due to their multidirectional health-promoting effects, dietary supplementation of probiotics and inulin is recommended for patients afflicted by a number of disorders, including chronic kidney disease (CKD). Namely, it has been demonstrated that a probiotics microorganism which needs urea, uric acid, and creatinine for its growth, enhance renal clearance of these metabolites (Ranganathan et al. 2010; Vitetta and Gobe 2013). Health-promoting effect of high content of inulin in the diet on the kidneys was also demonstrated in patients with type 2 diabetes mellitus. This type of diet applied to such patients reduces the risk of CKD (Krishnamurthy et al. 2012; Fujii et al. 2013). It has also been shown that dietary inulin indirectly affects kidneys alone. Rondón and co-workers (2008) showed that the supplementation of diet with 10% of inulin in mice resulted in the reduction of transient receptor potential melastin 6 (TRPM6) expression in the distal renal tubules. Rault-Nania and co-workers (2008) found that in rats with hypertension, a similar concentration of inulin to the diet prevented the characteristic volume increase of the proximal tubular cells. Both in humans and many animal species, the use of inulin in the diet and probiotics results in positive balance of micro- and macronutrients. Increasing the bioavailability in the gut and, in consequence, an increased concentration in the blood plasma of many minerals should therefore provoke appropriate changes in renal excretion of water. For as it is already known, in order to maintain stability of fluids and constant volume of extracellular fluid, changes in the concentration of the individual components should be accompanied by alterations in renal reasorption of water. Therefore, we decided to conduct a pilot study and test the hypothesis that in the kidneys of pigs fed with feed containing probiotics and inulin change the localization and increase the expression of aquaporin 2 (AQP2), a protein which plays an essential role in renal water reabsorption.

MATERIAL AND METHODS

Experimental Animals

All experiments were performed in accordance with the principles and procedures of Local Commission of Ethics for the Care and Use of Laboratory Animals (No. 30/2010 of 24.03.2010). The study was carried out on 16 Danbred x Duroc crossbred piglets (males). During the experiment, the animals were remained under unified and controlled environmental conditions. From the 10^{th} day of life the piglets were divided into 2 nutrition

groups (n = 8). Piglets from the control group were feed ad libitum, with the standard diet containing: wheat (45%), barley (20%), corn starch (5%), extruded full-fat soybeans (5.9%), dry sweet whey (9.7%), fish meal (4%), soybean oil (3.39%), calcium formate (0.3%), pasture chalk (0.5%), calcium phosphate (0.6%), pasture salt (0.07%), L-lysine (0.6%), DL-methionine (0.2%), L-threonine (0.25%), L-tryptophan (0.09%) and premix (0.4%).The animals from the experimental group were feed ad libitum, with standard diet supplemented with 0.05% probiotic and 2% water/alcohol inulin extract from chicory root (Grela et al. 2014). Probiotic (ŁAVIPAN) was composed of Lactococcus lactis IBB 500 minimum content of 10 x 9 CFU/g, Carnobacterium divergens S1 minimum content of 10 x 9 CFU/g, Lactobacillus casei ŁOCK 0915 minimum content of 10 x 9 CFU/g, Lactobacillus plantarum ŁOCK 0862 minimum content of 10 x 9 CFU/g, Sacharomyces cerevisiae ŁOCK 0862 minimum content of 10 x 9 CFU/g. The dose of probiotic was chosen in accordance with the manufacturer's recommendations. Inulin (HPX, Orafi) had polymerization degree higher or equal 23 and did not contain residue of other sugars. Piglets were sacrificed at the age of 50 days and kidneys were dissected. Obtained material was washed twice with ice-cold 0,9% NaCl solution and subsequently twice with ice-cold Krebs-HEPES buffer (20 mM, pH 7.4).

SDS – PAGE and Western blot

The tissue samples were placed in the lysis buffer (5M urea, 2M thiourea, 4% CHAPS, 40 mM Tris, 0.2% ampholytes pH 3–10, nuclease 1 : 1000) containing protease inhibitor cocktail 1 : 100 (Sigma-Aldrich). Afterwards, the tissue samples were frozen in liquid nitrogen and were homogenized using the Tissue Lyser, QIAGEN. The homogenates were centrifuged at 20.800 x g for 15 min at 4°C. The samples were warmed to 37°C and loaded on the 12% polyacrylamide gels and run for 120 min at 100 V. The proteins of studied gels were then electrotransferred (12V, 14 min) to PVDF membranes. The membranes were blocked with 5% non-fat milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with rabbit polyclonal antibodies anti-AQP2 H7661 (Department of Biomedicine and Anatomy, Aarhus University, Denmark) diluted 1 : 1000, followed by incubation with secondary anti-rabbit (120P Serotec) horseradish peroxidase-conjugated antibodies. The labeling was visualized by the enhanced chemiluminescence (ECL plus) system and exposure to CCD camera (Versadoc 4000MP, Bio Rad). The densitometry values and band optical density (OD) were evaluated with Quantity One software. Mean values and standard deviations were calculated. The resulting data were analysed by one- way ANOVA and Dunkan multiple range post hoc test (Statistyca, 10.0^{TM}) in order to test significance of differences. Expression of AQP2 was normalized against β-actin, which was used as an internal control.

Immunohistochemistry

The kidneys were fixed in 4% paraformaldehyde and embedded in paraffin blocks and sliced into 2–3 μm thick sections on a rotary microtome. Preparations were deparaffinized in xylene and ethanol with decreasing concentration, and were used for further hematoxylin/eosin (H&E), periodic-acid-Schiff (PAS) and immunohistochemical staining. In order to expose the epitopes, sections were twice boiled in a microwave oven (700W, 5 min)

in 10 nM citrate buffer (pH 6.0). Once cooled and washed with PBS, the slides were incubated for 60 min at room temperature with 2.5% BSA to block non-specific binding of the primary antibody. After that, the tissues were covered by the primary rabbit polyclonal anti-AQP2 H7661 antibody (Department of Biomedicine and Anatomy, Aarhus University, Denmark, final dilution 1 : 500). The incubation period lasted over the night in fridge (4°C). Next day, slides were washed in PBS and secondary antibody (goat anti-rabbit IgG labeled with Texas Red, Santa Cruse Biotechnology, sc-2780, final dilution 1 : 200) were placed on them to one hour. Sections were washed in distilled H_2O and closed with covering slip by medium with DAPI (Mounting Medium Ultra Cruz™, Santa Cruse Biotechnology, sc- 24941). Positive result of immunostaining was defined microscopically (confocal laser scanning microscope Olympus Fluoview FV 1000) by visual identification of red fluorescence. The expression of AQP2 was evaluated semi-quantitatively using grades: +, ++, or +++ (Table 1).

Table 1. Immunoexpression of aquaporin 2 (AQP2) in the principal cells of the kidney collecting ducts in growing piglets from control group and group fed diets supplemented with 0.05% probiotic and 2% water/alcohol inulin extract from chicory root
Tabela 1. Immunoekspresja akwaporyny 2 (AQP2) w komórkach głównych kanalików zbiorczych nerek prosiąt z grupy kontrolnej i z grupy żywionej paszą z dodatkiem 0.05% probiotyku i 2-procentowego wodno-alkoholowego roztworu inuliny z korzenia cykorii

Groups Grupy	Principal cells of the collecting duct Komórki główne kanalików zbiorczych		
	basolateral membrane błona podstawna	intracellular vesicles wewnątrzkomórkowe pęcherzyki	apical plasma membrane błona szczytowa
Control group Grupa kontrolna	+	+	++
Inulin and probiotics Inulina i probiotyki	+	+/++	+++

(+++) – strong – wysoka, (++) – moderate – średnia, (+) – weak expression – niska ekspresja.

RESULTS

Normal and typical structure of the renal cortex and medulla was observed in the preparations stained with H&E and PAS methods in all piglets studied (data not shown). There were no differences in renal histology between the animals from the control groups and the animals fed modified diet.

Figure 1 show the representative immunostaining of AQP2 in the renal tissue. AQP2 was found in the collecting ducts of renal medulla. In all tested groups of animals, AQP2 was mainly visible in the apical plasma membrane of the principal cells of the collecting duct. Weaker immunohistochemical staining was also visible in both the intracellular vesicles and basolateral membrane. Semi-quantitative evaluation of the AQP2 expression in the principal cells of the collecting duct is presented in Table 1. In the control group expression of AQP2 in the apical plasma membrane was moderate. However, in the pigs fed diet supplemented with inulin and probiotics AQP2 expression in apical plasma membrane increased. Expression of AQP2 increased also in the intracellural vesicles. In the control group expression of this protein was weak, while in the pigs fed modified diet was from weak to moderate.

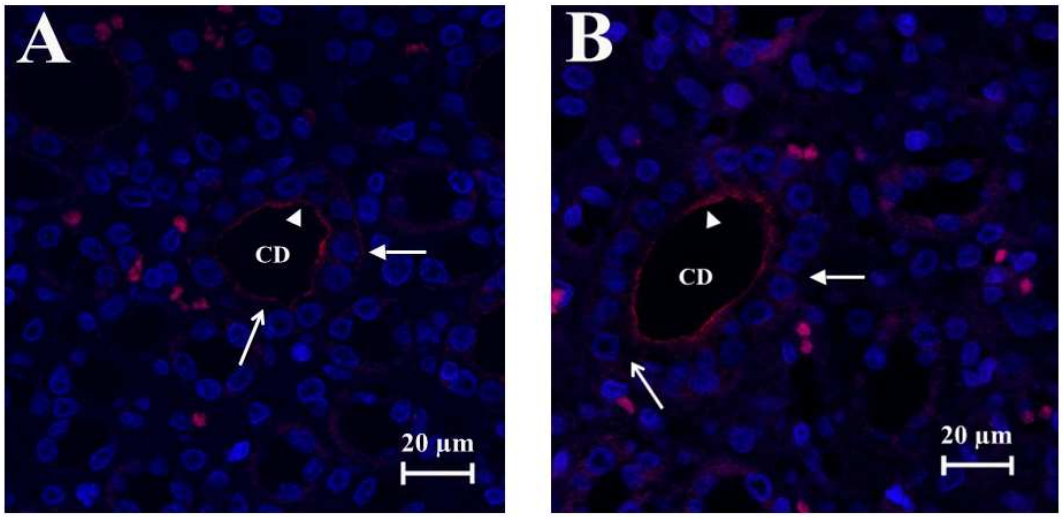

Fig. 1. Representative image of immunohistochemical staining of aquaporin 2 (AQP2) in paraffin-embedded sections of the renal medulla of growing piglets from control group (A) and group fed diets supplemented with 0.05% probiotic and 2% water/alcohol inulin extract from chicory root (B). Expression of AQP2 in the apical plasma membrane (white arrowhead), intracellural vesicles (white arrow) and basolateral membrane (white full headed arrow). CD – collecting duct
Ryc. 1. Reprezentatywny obraz immunohistochemicznego barwienia akwaporyny 2 (AQP2) w preparatach parafinowych rdzenia nerki prosiąt z grupy kontrolnej (A) i z grupy żywionej paszą z dodatkiem 0,05% probiotyku i 2% wodno-alkoholowego roztworu inuliny z korzenia cykorii (B). Ekspresja AQP2 w błonie szczytowej (biały grot), wewnątrzkomórkowych pęcherzykach (biała strzałka) i błonie podstawnej (strzałka z pełnym grotem). CD – kanaliki zbiorcze

Figure 2 show AQP2 abundance determined by Western blot. AQP2 antibodies recognized a 29 kDa band in protein samples from kidneys of growing piglets. It was confirmed, based on the analysis of average optical density of the bands, that animals fed a diet enriched with inulin and probiotics showed slight increased expression of AQP2 in the renal medulla of the kidneys.

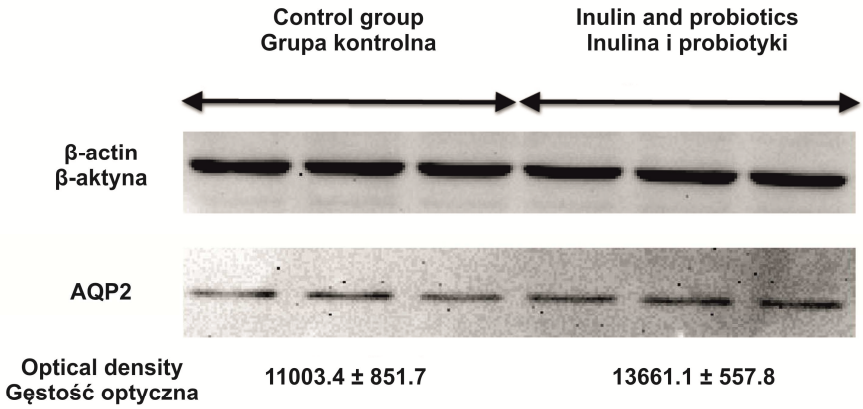

Fig. 2. Representative results of Western blot analysis of aquaporin 2 (AQP2) in the renal medulla of the control group and the piglets fed diets supplemented with 0.05% probiotic and 2% water/alcohol inulin extract from chicory root.
Ryc. 2. Reprezentatywny wynik analizy Western blot ekspresji akwaporyny 2 (AQP2) w rdzeniu nerek prosiąt z grupy kontrolnej i prosiąt z grupy żywionej paszą z dodatkiem 0,05% probiotyku i 2% wodno-alkoholowego roztworu inuliny z korzenia cykorii

DISCUSSION

AQP2 is one of 13 known mammalian isoforms of aquaporins, a family of small transmembrane proteins, selectively permeable to water and other small molecules, such as glycerol and urea (Fenton and Knepper 2007). AQP2 is mainly located in the apical plasma membrane and in the intracellural vesicles of the collecting ducts (CD) principal cells (Kim et al. 2005; Noda and Sasaki 2005). A small amount of this protein can also be found in the basolateral membrane of these cells (Nielsen et al. 1993). Specific constitution of this protein and its selective permeability only to water causes that AQP2 plays an important role in the renal water reabsorption and in the process of hypertonic urine production (Sasaki 2012). In the present study, IHC and Western blot analyses revealed that AQP2 in growing piglet is expressed in the principal cells of collecting ducts. The location, distribution and expression of AQP2 observed in piglets was typical and characteristic of many other animal species as well as in human (Nielsen et al. 1993; Kishore et al. 1996; Loffing et al. 2000; Bauchet et al. 2011; Mobasheri et al. 2011; Michałek et al. 2014).

It is widely known that AQP2 expression in mammalian kidneys is mainly regulated by vasopressin (AVP). This anti-diuretic brain secreted hormone is released from the posterior pituitary gland mainly due to the increase in the osmotic pressure of the extracellular fluid, and reduction of the circulating blood volume (Sasaki 2012). Vasopressin regulates water permeability in the kidney by two different processes. The first one is a short-term regulation, which occurs over a period of minutes as a result of the regulation of trafficking of intracellular vesicles containing AQP2 to and from apical plasma membrane. The second process is a long-term regulation, which occurs within hours or days as a result of the regulation of whole-cell AQP2 abundance (Wilson et al. 2013). In the short-term regulation, shuttling of AQP2 between intracellular vesicles and apical plasma membrane requires a functional AVP – AC – cAMP – PKA signaling cascade (Boone and Deen 2008). Binding of AVP to vasopressin type 2 receptor (V2R) causes an activation of G_s protein alpha subunit, which in turn stimulates two adenylate cyclases (AC), type III and VI.

As a result, increase production of the intracellular cyclic adenosine monophosphate (cAMP) and the activation of protein kinase A (PKA). In response to an increase in the concentration of cAMP, other kinases are activated, which are most likely to be involved in the process of epithelium permeability changes in the collective duct, ie. protein kinase B (PKB), serum/glucocorticoid regulated kinase (Sgk), myosin light-chain kinase and calmodulin--dependent kinases (CaM kinases) (Wilson et al. 2013). The active form of PKA phosphorylates Ser256, which is located in the cytoplasmic C-terminal region of AQP2 monomers. Phosphorylation of at least three AQP2 monomers in each tetramer is required to start AQP2 translocation from the intracellular vesicles and to fuse this protein with apical plasma membrane, thereby resulting in water reabsorption by the cell. After fusion with the cell membrane, AQP2 is excreted into urine or undergoes endocytosis (Moeller and Fenton 2012). According to Brown and colleagues (2008), the amount of AQP2 in the apical plasma membrane is a result of a balance between continuing endocytosis and exocytosis of AQP2, both in the presence and absence of AVP. The process of long-term regulation of the total amount of AQP2 in the principal cells of the collecting duct is the results of a balance between the

production of AQP2 by translation and removal from the cell by either degradation or exosomal secretion into urine (Wilson et al. 2013). As expected, the piglets fed the modified diet showed increased AQP2 expression mainly in the apical plasma membrane, as compared to the control animals. It is also interesting that the Western blot experiment revealed a slight increase in total AQP2 expression in the renal medulla. It can be concluded that the increased abundance of AQP2 in the apical plasma membrane and the total amount of this protein in the renal medulla, caused an increased renal water reabsorption in animals fed the modified diet.

Inulin is fermented in the large intestine and stimulates the growth of lactic acid bacteria, mainly from the genus Bifidobacterium. Additionally, in our experiment a probiotic was co-administered with inulin, which contained also Lactobacillus. Changes in gut microbiota, caused by the presence of inulin and probiotic, probably gave rise to the so-called bifidogenic effect, which is associated with selective fermentation of fructans by Bifidobacterium (Gibson 1995). As a result of the bifidogenic effect, there is a significant increase in the production of short chain fatty acids (SCFA) (Mair et al. 2010).

The result of increased production of SCFA is a decrease of pH of intestinal content. Low pH in the intestine not only reduced the proliferation of pathogenic microorganisms, but also increases the solubility of micro- and macronutrients, increases the pool of ionized components, and thus their absorption. In addition, SCFA can form complexes with minerals, which also increases their resorption in the intestine (Coudray et al. 2005). In pigs fed with diets supplemented with inulin, intestinal absorption of such elements as Fe, Mg, Zn, Mn, Zn or Cu is increased (Yasuda et al. 2009; Jolliff and Mahan 2012; Untea et al. 2013). Therefore, an increase in the plasma concentration of many mineral components, as a result of higher absorption in the gut, to maintain the current, correct water and electrolyte balance, must be accompanied by an increase in the resorption of water in the renal tubules. We speculate that increase expression of AQP2 mainly in the apical plasma membrane of principal cells and slight increase of the total amount of this protein in the renal medulla observed in pigs fed with diets supplemented with inulin and probiotics, enables renal reabsorption of water necessary to maintain a proper water-electrolyte balance, which links with our previously study (Michałek et al. 2016) and with the results obtained by Rondon and coworkers (2008). Rondon and colleages reported that mice fed a diet enriched with inulin showed reduced renal expression of TRPM6.

This protein transports Mg^{2+} across the apical membrane of the epithelial cells in kidney and large intenstine. The reduction in TRPM6 expression observed in renal tubules of the mice was explained by the authors as a homeostatic response of the kidney to the increased Mg^{2+} absorption in the gut. Namely, in order to maintain the proper magnesium balance, clearance of this mineral was increased through downregulation of TRPM6. We belive in the present study we also observe homeostatic response of the kidney and increased renal water reabsorption caused by intensify intestinal absorption of many components. Similar results we have also obtained in our previously study (Michałek et al. 2016). In the kidney of pigs fed diet supplemented with different levels of inulin type fructans we have observe statistical increased a total AQP2 in the renal medulla and increased expression of this protein in apical plasma membrane and intracellular vesicles of principal cells.

CONCLUSION

In conclusion we have demonstrated that in Danbred x Duroc crossbred growing piglets AQP2 is expressed in the principal cells of the collecting duct as was excepted. Localization of this protein in the particular parts of these cells (apical and basolateral plasma membrane and intracellular vesicles) is typical and characteristic for both humans and other species. Moreover, the presented study revealed that supplementation of feed for growing piglets with inulin and probiotics resulted in an increased expression of this protein in the apical plasma membrane and in a slight increase in the total amount of AQP2. We can speculate that the probable cause of change in immunolocalization and increased expression of AQP2 in the kidneys of pigs studied was higher demand for water relative to the positive balance of many mineral components. The increase in renal expression of AQP2 seems to be a positive effect of diet supplementation with inulin and probiotics. Nevertheless, a more comprehensive explanation of this effect requires further, more in-depth studies involving other specific parameters of renal function. We do hope, however, that the results of this preliminary study will contribute to future research, will inspire interest in porcine renal AQP2 and will have a part in the advances of the knowledge in this area and the species.

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Abstract. Feeds supplemented with probiotics and inulin, which help in the maintenance of desired intestinal microbiota, have beneficial, multidirectional, health-promoting effects. One such effect involves increased bioavailability and absorption of macro- and micronutrients. Enhanced intestinal absorption of many minerals and, in consequence, their elevated blood plasma concentration should provoke a homeostatic response of the kidneys in order to regulate water excretion. Therefore, we have undertaken a pilot study to test the hypothesis that probiotics and inulin added to feed change the localization and increase the expression of aquaporin 2 (AQP2), a protein essential in renal water reabsorption. The study was carried out on 16 Danbred x Duroc crossbred piglets (males). Based on immunohistochemistry (IHC) results, we found that the abundance of AQP2 in animals fed with the modified diet increased mainly in the apical plasma membrane of the collecting duct principal cells. Western blot analysis revealed that in animals fed the supplemented diet the total AQP2 expression in the renal medulla increased too. These changes in the location and expression of AQP2 imply increased renal water reabsorption. Such increased water reabsorption in response to the positive balance of many components seems to support the evidence of the positive effects of dietary probiotics and inulin.