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Regulation of metabolic processes by nutritional factors in chicken

PhD thesis

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List of abbreviations

4E-BP1	4E-binding protein 1
acetyl~CoA	acetyl~coenzyme A
Akt	protein kinase B
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
AOAC	Association of Analytical Chemists
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BSA	bovine serum albumin
But	n-butyrate
cAMP	cyclic adenosine monophosphate
cDNA	cyclic deoxyribonucleic acid
CFU	colony-forming unit
CK	creatine kinase
CP	crude protein
CSA	Campylobacter selective agar
Ctr	control
CYP	cytochrome P450 enzyme
d	day
DNA	deoxyribonucleic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCGR	glucagon receptor
GIP	glucose-dependent insulinotropic polypeptide, gastric inhibitory polypeptide
GLP-1	glucagon-like peptide 1
GLUT	glucose transporter
GPCR	G-protein-coupled receptor
GPR41, GPR43, GPR109A	G-protein receptor 41, 43 and 109A
$G_s\alpha$, Gq	G-protein sα and q subunits
H^+	hydrogen cation
H2A, H2B, H3, H4	histone core protein 2A, 2B, 3 and 4
HAT	histone acetyl transferase
HDAC	histone deacetylase
IRS-1 (IRS-1_a)	insulin receptor substrate 1 (“a” lowercase subscript = activated)

IRα	insulin receptor α subunit
IRβ	insulin receptor β subunit
LP	lowered protein
MAP-kinase	mitogen-activated protein kinase
MB	maize-based diet
MBC	minimum bactericidal concentration
MCT	monocarboxylate transporter
MIC	minimum inhibitory concentration
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
mTOR	mammalian (or mechanistic) target of rapamycin
NP	normal protein
NSP	non-starch polysaccharide
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween 20
PI3K ($PI3K_a$)	phosphatidylinositol-3-kinase (“a” lowercase subscript = activated)
PIP_2	phosphatidylinositol diphosphate
PIP_3	phosphatidylinositol trisphosphate
PKA	protein kinase A
pKa	the negative logarithm of the acid dissociation constant
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PPARα	peroxisome proliferator activated receptor alpha
q-PCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
S6K	p70 ribosomal S6 kinase
SCFA	short chain fatty acid
SE	standard error
SEM	standard error of mean
SGLT	sodium-glucose co-transporter
SMCT	sodium-dependent monocarboxylate transporter
TG	triglyceride
TP	total protein
WB	wheat-based diet

1. Summary

The promotion and maintenance of metabolic health with optimal feed utilization is of special importance in the broiler industry to ensure intensive and economic growth and simultaneously to improve animal welfare, as these aspects determine the quality and quantity of the endproducts. The restricted application of antibiotics and hormones in the European Union turned the scientific and public interest towards the growth promoting beneficial effects of alternative feed additives, especially the most commonly used short-chain fatty acid n-butyric acid or its anion form n-butyrate (in the followings butyrate) in order to reach the above mentioned goals. Butyrate can be used as feed additive in the form of its free salts, or applied in protected forms, but it also can be the endproduct of the endogenous anaerobe microbial fermentation in the large intestines, promoted by bypass carbohydrates, e.g. non-starch polysaccharides (NSPs) in the fodder. The NSPs serve as substrates for the short chain fatty acid, and predominantly butyrate synthesis, enabling this molecule to exert its widespread intestinal and extraintestinal effects, resulting in better growth performance and intestinal health. Although butyrate supplementation of the diet is getting common in pig and poultry nutrition, there are still open questions and contradictory data especially under special feeding conditions. Therefore, the aim of this PhD study was to investigate certain age-dependent general and tissue-specific effects of butyrate of exogenous and endogenous origin in combination with distinct crude protein content of the diet in chicken. In addition, we also intended to test the theoretical antimicrobial efficacy of butyrate against various *Campylobacter jejuni* strains in an *in vitro* assay.

The hypothesis of the *in vivo* experiments was that distinct sources of butyrate, as well as dietary crude protein content might affect the avian metabolism differently, or can have different action when applied in combination. Therefore, apart from sodium (n-)butyrate supplementation of the feed in the commonly used dose (1.5 g/kg diet), two cereals, wheat and maize – wheat containing c.a. tenfold more NSPs than maize – were chosen as bases of the experimental diets (WB vs. MB groups). Further, groups with recommended and slightly reduced crude protein content (normal protein [NP] and low-protein [LP] groups, the latter reduced by 15% and completed with limiting amino acids) were formed to gain information on the effects of this ecologically beneficial rearing technology of growing interest.

The responsiveness of selected markers of the metabolism of nitrogen-containing compounds, glucose and lipid metabolism, as well as insulin homeostasis were monitored on Ross 308 broilers. To follow the suspected age-dependency, body weight measurements were performed and peripheral blood samples were gained at the age of 7, 21 and 42 days (d). Plasma total protein (TP) concentration increased in WB and decreased in LP groups on d 21, while butyrate reduced albumin/ TP ratio on d 7. Uric acid level was elevated by WB diet on d

7 and 21, and by butyrate on d 21, but decreased in LP groups on d 21 and 42. Aspartate aminotransferase activity was increased by WB diet on d 21, and LP diet intensified creatine kinase activity on d 21 and 42. Moreover, the activity of this enzyme elevated c.a. 13-fold until d 42, compared to d 7 measurements, independent of diet composition. Blood glucose level decreased, but triglyceride concentration changed opposite in WB groups on d 21. No diet-induced, but age-dependent changes of glucagon-like peptide 1 and insulin concentrations were observed. Additionally, LP diet significantly increased body weight of the birds at all sampling points, while WB diet had the same growth promoting effect on d 21 and 42, serving as background data for the examinations. In conclusion, the chickens showed outstanding ability to respond to the nutritional factors in the phase of intensive growth (d 21), and WB diet proved to be the most potent in altering metabolic processes, presumably via intensified butyrate production due to the manifold higher NSP content of wheat, compared to maize. However, all but one parameters were in the physiological range, suggesting that the applied dietary strategies might be safe in poultry farming.

Changes in the carbohydrate homeostasis might lead to better insulin sensitivity and thus growth of birds, therefore, certain key members of the hepatic insulin and glucagon signaling was also investigated. Considering the notable age-dependent changes of the avian metabolic responsiveness, liver samples of 21-day-old chickens were subjected to quantitative polymerase chain reaction (q-PCR) and Western blot analyses in order to study the gene expression and protein abundance pattern of glucagon receptor (GCGR), insulin receptor beta (IRβ) and mammalian (also known as mechanistic) target of rapamycin (mTOR). Hepatic GCGR and mTOR gene expression were up-regulated by WB and LP diet. GCGR and IRβ protein abundance decreased in groups with butyrate supplementation; however, the quantity of IRβ and mTOR proteins increased in WB groups. Based on these data, the applied dietary strategies may be useful tools to modulate hepatic insulin and glucagon signaling of chickens in the period of intensive growth. The obtained results might contribute to the better understanding of glycemic control of birds, provide an opportunity of improving insulin sensitivity, hence, the production parameters and the welfare of broilers, further, might serve new information for animal protection technologies aiming the welfare of broiler chickens.

In order to evaluate the result of metabolic changes from practical point of view, the effect of unprotected and different types of protected butyrate in combination with the already described dietary crude protein levels was also studied, with conventional maize-based diets. At the age of 42 days, the weight of carcass traits and several organs were measured, and the chemical composition of pectoral and femoral muscles was analyzed. Carcass weight significantly increased as the effect of LP diet and all protected butyrate types tested, while the relative breast meat yield was significantly higher in the LP than in the NP groups and in both unprotected and protected butyrate-supplemented chickens compared to controls. The weight

of liver augmented in LP groups, and the relative abdominal fat mass tended to be reduced by the unprotected form of butyrate only. The protein content of the femoral muscle was significantly decreased, but its lipid content changed opposite by the LP diet and by all types of butyrate addition. However, no changes were detected in the chemical composition of pectoral muscle. Concluding our results, breast meat production can be effectively stimulated by reducing dietary crude protein content with limiting amino acid addition and by applying any form of butyrate as feed additive, while its chemical composition remains unchanged, in contrast to the femoral muscle. The aforementioned nutritional strategies seem to be proper tools to increase carcass yield and to selectively alter meat composition of broilers, contributing to more efficient poultry meat production.

Not only satisfactory amount, but safe meat production is also fundamental purpose of the poultry industry. Since *Campylobacter jejuni* (*C. jejuni*) is one of the major food borne zoonotic pathogens transmitted by chicken meat, there is a high demand on intervention strategies reducing intestinal *Campylobacter* colonization and carcass contamination. Due to its selective antimicrobial property, butyrate may be a useful tool in *Campylobacter* controlling programs. In our *in vitro* study the antibacterial efficacy of sodium (n-)butyrate on eight *C. jejuni* strains were investigated at two pH values (6.0 and 7.4), to explore the sensitivity of different strains. The *C. jejuni* strains were incubated in Bolton broth buffer with different concentrations of sodium butyrate for 48 hours, then minimum inhibitory and minimum bactericidal concentrations (MIC and MBC) of butyrate were determined by colony counting on Campylobacter selective agar plates. Butyrate proved to exert its inhibitory and bactericidal effect only in 100 mmol/l concentration at pH 7.4, while at pH 6.0, its efficiency increased to 5 mmol/l as MIC and MBC was measured strain-dependently as 5 or 7.5 mmol/l. All strains except one showed similar sensitivity to butyrate. Decreased butyrate susceptibility of this single field isolate was associated with ampicillin resistance as well. A nutritionally achievable concentration of butyrate was found to act effectively against most *C. jejuni* strains *in vitro* at lower pH, hence it might be a useful tool to reduce enteral *C. jejuni* colonization. However, several additional factors might influence butyrate's antibacterial efficacy under *in vivo* conditions, which should be taken into consideration.

1. Összefoglalás

A brojlerágazatban az anyagcserefolyamatok egészséges működésének elősegítése és fenntartása az optimális takarmányhasznosítással együtt különösen fontos az intenzív és gazdaságos növekedés biztosítása érdekében, egyszersmind az állatok jólétének figyelembevételével, mivel ezek a szempontok meghatározzák a végtermék minőségét és mennyiségét. A fent említett célok elérése érdekében az antibiotikumok és hormonok alkalmazásának korlátozása az Európai Unióban a tudomány és a közvélemény érdeklődését az alternatív takarmány-adalékanyagok, különösen a leggyakrabban használt rövid szénláncú zsírsav, az n-vajsav vagy anionformája, az n-butirát (a továbbiakban butirát) növekedést elősegítő jótékony hatásai felé fordította. A butirát takarmány-adalékanyagként használható szabad sói, vagy különféle védett formáiban, de lehet a vastagbélben végbemenő endogén anaerob mikrobiális fermentáció végterméke is, mely utóbbi folyamatot a takarmányban lévő bypass-szénhidrátok, pl. nem keményítő típusú poliszacharidok (NSP-k) jelenléte segít elő. Az NSP-k szubsztrátként szolgálnak a rövid szénláncú zsírsav, elsősorban a butirát szintéziséhez, lehetővé téve e molekula széles körű bélrendszerben és azon kívül kifejtett hatását, ami jobb növekedési erélyt és egészségesebb bélrendszert eredményez. Bár a sertés- és baromfitakarmányozásban egyre gyakoribb a takarmány butirátkiegészítése, még mindig vannak nyitott kérdések és ellentmondásos adatok, melyek különösen speciális takarmányozási körülmények között fordulnak elő. Ezért doktori munkám fő célja az volt, hogy az exogén és endogén eredetű butirát bizonyos korfüggő általános és szövetspecifikus hatásait vizsgáljam a takarmány különböző nyersfehérje-tartalmával kombinálva csirkében. Emellett, a butirát antimikrobiális hatékonyságát is tesztelni kívántam különböző *Campylobacter jejuni* törzsekkel szemben *in vitro* vizsgálatokban.

Az *in vivo* kísérletek alapfeltevése az volt, hogy a különböző butirát források, valamint a táplálék nyersfehérje-tartalma eltérő módon befolyásolhatja a madarak anyagcseréjét, illetve kombinációban alkalmazva eltérő hatást fejthetnek ki. Ezért a takarmány általánosan használt dózisban (1,5 g/kg takarmány) történő nátrium(n-)butirát kiegészítése mellett két gabonafélét, búzát és kukoricát - a búza kb. tízszer több NSP-t tartalmaz, mint a kukorica - választottunk a kísérleti takarmányok alapjául (WB vs. MB csoportok). Továbbá, ajánlott és enyhén csökkentett nyersfehérje-tartalmú csoportokat (normál [NP] és alacsony fehérjetartalmú [LP] csoportok, utóbbi 15%-kal csökkentett és limitáló aminosavakkal kiegészített) alakítottunk ki, hogy információt nyerjünk ez utóbbi, ökológiailag előnyös és egyre nagyobb érdeklődésre számot tartó tenyésztési technológiának a hatásairól.

Ross 308 brojlereken végzett kísérletben a nitrogéntartalmú vegyületek anyagcseréje, a glükóz- és lipidanyagcsere, valamint az inzulin homeosztázis kiválasztott markereinek válaszkészségét követtük nyomon. A feltételezett korfüggés nyomon követése érdekében

testtömeg-méréseket végeztünk, és perifériás vérmintákat vettünk 7, 21 és 42 napos korban. A plazma összfehérje (TP) koncentrációja a WB csoportokban nőtt, az LP csoportokban csökkent a 21. napon, míg a butirát az 7. napon csökkentette az albumin/ TP arányt. A húgysavszintet a búza alapú takarmány az 7. és a 21. napon megemelte, ugyanígy a butirát a 21. napon, az LP-csoportokban azonban a 21. és a 42. napon csökkentette. Az aszpartát-aminotranszferáz aktivitás a 21. napon nőtt a WB csoportokban, a csökkentett nyersfehérje-tartalmú, limitáló aminosavakkal kiegészített táp pedig fokozta a kreatin-kináz aktivitást a 3. és a 42. napon. A WB-csoportokban a vércukorszint csökkent, de a triglicerid koncentráció ellentétesen változott a 21. napon. A glükagon-szerű peptid 1 és az inzulin koncentrációjának csupán életkorfüggő változását figyeltük meg. Emellett az LP-takarmány minden mintavételi időpontban jelentősen növelte a madarak testtömegét, míg a búza alapú takarmány a 3. és a 42. napon ugyanilyen növekedésserkentő hatással bírt, mely adatok háttérinformációként szolgáltak a vizsgálatokhoz. Összefoglalva, a csirkék az intenzív növekedés szakaszában (21. nap) kiemelkedő válaszkészséget mutattak a takarmányozási tényezőkre, és a búza alapú takarmány bizonyult a leghatásosabbnak az anyagcsere-folyamatok megváltoztatásában, feltehetően a kukoricához viszonyítva sokszorosan magasabb NSP-tartalma által kiváltott, intenzívebb butirát termelés révén. Ugyanakkor, egy kivétellel, minden vizsgált paraméter a fiziológiás tartományba esett, ami arra utal, hogy az alkalmazott takarmányozási stratégiák biztonságosak lehetnek a baromfitenyésztésben.

A szénhidrát-homeosztázisban bekövetkező változások fokozott inzulinérzékenységhez és ezáltal a madarak intenzívebb növekedéséhez vezethetnek, ezért a máj inzulin- és glükagon-jelpályájának egyes kulcsfontosságú tagjait is vizsgálni kívántuk. Tekintettel a madarak anyagcseréjének figyelemre méltó, életkorfüggő változásaira, 21 napos csirkék májmintáit kvantitatív polimeráz láncreakció (q-PCR) és Western blot analízisnek vetettük alá a glükagon receptor (GCGR), az inzulin receptor béta (IRβ) és a mammalian (más néven mechanistic) target of rapamycin (mTOR) gén- és fehérjeexpressziós mintázatának tanulmányozása érdekében. A hepatikus GCGR és mTOR génexpresszió WB és LP takarmányozás hatására fokozódott. A GCGR és az IRβ fehérje mennyisége csökkent a butirát-kiegészítésben részesülő csoportokban; az IRβ és az mTOR fehérjék mennyisége azonban nőtt a WB csoportokban. Ezen adatok alapján az alkalmazott takarmányozási stratégiák hasznos eszközök lehetnek a csirkék hepatikus inzulin- és glükagon-jelpályájának befolyásolására az intenzív növekedés időszakában. A kapott eredmények hozzájárulhatnak a madár vércukorszint szabályozásának jobb megértéséhez, lehetőséget biztosítanak az inzulinérzékenység, ezáltal a termelési paraméterek javítására madarakban, továbbá újabb adatokat szolgáltathatnak a brojlercsirkék jólétét célzó állatvédelmi technológiák számára.

Az anyagcsere-változások eredményének gyakorlati szempontból történő értékelése érdekében a nem védett és a különböző típusú védett butirát hatását a már ismertetett

takarmány nyersfehérje-szintekkel kombinálva is vizsgáltuk, hagyományos, kukoricaalapú takarmányokat alkalmazva. Negyvenkétnapos korban megmértük a karkassz, egyes húsrészek és több szerv tömegét, valamint elemeztük a mellizom és a combizom kémiai összetételét. A karkassz tömege szignifikánsan nőtt az LP- takarmány és minden vizsgált védett butirát-típus hatására, míg a mellhús kihozatal relatív értéke az LP csoportokban, a nem védett és védett butiráttal kiegészített csoportokban egyaránt szignifikánsan magasabb volt a kontrollhoz képest. A máj tömege az LP csoportokban növekedett, a relatív hasi zsírtömeget pedig csak a nem védett butirát csökkentette trendszerűen, de statisztikailag nem szignifikánsan. A combizom fehérjetartalma szignifikánsan csökkent, de lipidtartalma ezzel ellentétben növekedett az LP-takarmány és a butirát-kiegészítés minden típusa hatására, a mellizom kémiai összetételében azonban nem észleltünk változást. Eredményeinket összegezve, a mellhús tömege hatékonyan növelhető a takarmány nyersfehérje-tartalmának csökkentésével (limitálóaminosav-kiegészítéssel) és a butirát bármely formájának takarmány-adalékanyagként történő alkalmazásával, miközben a combizomtól eltérően a kémiai összetétele változatlan marad. A fent említett takarmányozási stratégiák megfelelő eszközöknek tűnnek a karkassz hozamának növelésére és a brojlercsirkék húsösszetételének szelektív megváltoztatására, hozzájárulva a hatékonyabb baromfihús termeléshez.

Nemcsak a kielégítő mennyiségű, hanem a biztonságos hústermelés is alapvető célja a baromfiiparnak. Mivel a *Campylobacter jejuni* (*C. jejuni*) a csirkehús által terjesztett egyik legfontosabb élelmiszer-eredetű, zoonotikus kórokozó, nagy szükség van a bélbeli *Campylobacter*-kolonizációt és a karkassz szennyezettségét csökkentő beavatkozási stratégiákra. Szelektív antimikrobiális tulajdonsága miatt a butirát hasznos eszköz lehet a *Campylobacter*-eradikációs programokban. *In vitro* vizsgálatunkban a nátrium(n-)butirát antibakteriális hatékonyságát tanulmányoztuk nyolc *C. jejuni* törzsre két pH értéken (6,0 és 7,4), hogy feltárjuk a különböző törzsek érzékenységét. A *C. jejuni* törzseket különböző koncentrációjú nátrium-butirátot tartalmazó Bolton-levesben inkubáltuk 48 órán keresztül, majd a butirát minimum gátló és minimum baktericid koncentrációját (MIC és MBC) Campylobacter-szelektív agarlemezeken telepszámlálással határoztuk meg. A butirát csak 100 mmol/l koncentrációban fejtette ki gátló és baktericid hatását pH 7,4 értéken, míg pH 6,0 értéken hatékonysága 5 mmol/l-re nőtt (MIC), és az MBC törzsfüggően 5 vagy 7,5 mmol/l-nek adódott. Egy kivételével valamennyi törzs hasonló érzékenységet mutatott a butirátra. Ennek az egyetlen telepi mintából származó törzsnek a csökkent butirátérzékenysége ampicillinrezisztenciával is társult. A butirátnak megfelelő takarmányozással *in vivo* is kialakítható olyan koncentrációja, amely *in vitro* alacsonyabb pH-n a legtöbb *C. jejuni* törzzsel szemben hatékonynak bizonyult, ezért hasznos eszköz lehet az enterális *C. jejuni* kolonizáció csökkentésére. Azonban számos további tényező befolyásolhatja a butirát antibakteriális hatékonyságát *in vivo* körülmények között, amelyeket feltétlenül figyelembe kell venni.

2. Introduction and literature overview

The constantly growing human population shows an increscent demand for satisfactory amount of healthy and economic meat production, fulfilling animal welfare considerations as well. Regarding that broiler meat is one of the most easily available, affordable, and therefore the most common protein source in human nutrition, improving the efficiency of poultry farming has an increasing significance worldwide. Since the legislation in the European Union restricted the application of antibiotics and hormones in 2006 (ordered by the regulation of no. 1831/2003/EC on additives for use in animal nutrition; Phillips 2007), scientific attention turned towards seeking novel possibilities for enhancing growth promotion. Alternative strategies, such as optimized nutrient composition of feed and application of feed additives, especially the use of short chain fatty acids (SCFAs) are apparently promising tools on this field, where the salts of (n-)butyric acid (in the followings: butyrate, regardless of its dissociation state) have outstanding importance and become increasingly popular in livestock industry.

2.1. Short chain fatty acids in general

The SCFAs are monocarboxylic acids containing fewer than six carbon atoms, existing either in straight-chained or branched forms. They are the major end products of the anaerobe microbial fermentation of indigestible or unabsorbed carbohydrates and to a lesser extent, of amino acids in the gastrointestinal tract. In this active symbiotic process, compounds indigestible by mammalian and avian enzymes serve as substrates for the gut microbiota. In turn, 95% of the produced SCFAs can be absorbed and utilized by the host cells, exerting regulatory effects as well (den Besten et al. 2013). The major sites of SCFA synthesis are the forestomaches of ruminants and the large intestines (particularly the cecum and the proximal colon) of monogastric mammals, birds and human (Bergman 1990), where the total SCFA concentration can reach 70-140 mmol/l (den Besten et al. 2013). Further, in case of appropriate diet and bacterial composition, SCFA production also occurs in the small intestines, but its intensity is negligible, compared to that in colon (Smiricky-Tjardes et al. 2003; Rehman et al. 2007; den Besten et al. 2013).

The most abundant members of SCFAs are acetic acid (C2), propionic acid (C3) and n-butyric acid (C4), representing 90-95% of SCFAs present in the colon (Cook and Sellin 1998; Ríos-Covián et al. 2016), also called acetate, propionate and n-butyrate (in the followings butyrate) when dissociated. Their molar ratio varies from 75:15:10 to 40:40:20 in a healthy animal (Bergman 1990), but these proportions highly depend on the combination of several dietary circumstances, including amount and quality of available substrates, composition of inhabitant microorganisms, transit time and pH of the ingesta (Wong et al. 2006).

Dietary fiber mainly consists of lignin and non-starch polysaccharides (NSPs), the latter constituting the major fraction of cell wall polysaccharides (Sethy et al. 2015). The NSPs are divided to soluble and insoluble fractions, and composed of macromolecular polymers of monosaccharides linked by glycosidic bonds (Hesselman 1989). NSPs include cellulose, hemicellulose, pectins, glucans, gums, mucilages, inulin and chitin. The most abundant NSPs derived from cereal grains are hemicellulose xylans and arabinoxylans, as well as β-glucans and cellulose (Sethy et al. 2015). Moderately high levels of soluble NSPs, especially arabinoxylans exert some adverse effects on digestion by increasing the viscosity of digesta (Cowan et al. 1996). Higher viscosity decreases passage rate and ensures more time for bacteria to thrive (de Lange 2000), but these undesirable effects can partly be eliminated by the enzyme supplementation of NSP-rich (e.g. barley, rye or wheat-based) diets (Kiarie et al. 2014; Molnár et al. 2015). NSP-degrading enzymes, such as xylanase and glucanase facilitate the degradation of long-chained NSPs into shorter oligosaccharides, thus reduce viscosity and emphasize the prebiotic properties of soluble NSPs against their disadvantages by providing more fermentable oligosaccharides for probiotic bacteria (de Lange 2000; Jamroz et al. 2002). Molnár et al. (2015) have also shown that wheat-based, NSP-rich diet with enzyme addition supported total SCFA and butyrate production in the cecum of chickens. Further, several studies reported fibre (thus NSP)-rich diet to influence insulin homeostasis in mice (Miyamoto et al. 2018), chicken (Kulcsár et al. 2016) and human (Boll et al. 2016).

The vast majority of SCFAs is derived from the anaerobe microbial fermentation of soluble NSPs (Flint et al. 2008), while valine, leucine and isoleucine amino acids can serve as precursors of branched-chain SCFAs, contributing up to 5% of the total SCFAs (Ríos-Covián et al. 2016). In the process of the anaerobe fermentation of indigestible nutrients, long chained poly- and oligosaccharides are cleaved to shorter compounds, then to monosaccharides. Further, these monosaccharides are degraded intracellularly into pyruvate in the glycolysis (Miller and Wolin 1996), serving as precursor for the production of distinct SCFAs. Acetate can be formed either by the decarboxylation of pyruvate or in some bacteria, utilizing CO_2 and H_2. Propionate production has three pathways, namely propanodiol, succinate and lactate pathway. The synthesis of butyrate starts with the condensation of two acetyl~coenzyme A (acetyl~CoA) molecules, and the final enzymatic step is determined by the type of bacteria producing it (Ríos-Covián et al. 2016).

Despite the lowest proportion in the gut, butyrate has the most remarkable biological activity and effects on the intestinal microbiota and on the host itself (Guilloteau et al. 2010). Its growth promoting action was demonstrated in several species (Gálfi and Bokori 1990; Hu and Guo 2007), bringing it to the fore as feed additive in pig and poultry nutrition.

2.2. General properties of the 4-carbon short chain fatty acid butyrate

The fourth member of the homologous line of the non-branched SCFAs is the (n-)butyric acid. On room temperature, it is an oily, colorless liquid with unpleasant scent, easily dissolvable in water, ethanol and ether as well. It can be considered as a weak acid with a pKa value of 4.82, meaning that it remains undissociated (butyric acid) below and turns to dissociated (butyrate) form above this pH. In the followings, the molecule is referred as "butyrate" regardless of its dissociation state.

Due to its numerous beneficial intestinal and extraintestinal effects, butyrate is of special interest as feed additive particularly in pig and poultry nutrition, mixed into the fodder as free salts of butyrate anion (the most frequently sodium or calcium salts) or applied in protected forms (Chamba et al. 2014). In chicken the absorption of free butyrate salts starts in the crop and is completed in the gizzard, therefore, negligible amount of butyrate reaches the small intestines (Kulcsár et al. 2017). Protection methods include esterification (usually with glycerol), a special film-coating process using carbohydrate or fat matrix, or combination of these methods, resulting film-coated, fat-embedded and micro-encapsulated types of protected butyrate. Protection of the molecule ensures prolonged release of butyrate: butyric acid glycerides provide a lipase-driven butyrate release in the proximal small intestines (Antongiovanni et al. 2007), but other types proved to reach even large intestinal sections (Moquet et al. 2016; Kulcsár et al. 2017). On the other hand, protection might facilitate the application and manufacturing of the product by reducing butyrate's characteristic odor and by improving blending properties.

Owing to its pKa value 4.82, butyrate remains undissociated in the proximal sections of the gastrointestinal tract, e.g. stomach of monogastric mammals and proventriculus and gizzard of poultry, where the pH is strongly acidic (~pH 1-3). As the cell membrane is permeable for this undissociated form, butyrate enters the intracellular space by simple diffusion, where it dissociates to butyrate anion plus hydrogen cation (H^+), and therefore gets captured on the pH of the cytoplasm higher than its pKa value. This phenomenon ensures a highly effective absorption of butyrate (and SCFAs in general) from this proximal part of the intestinal tract (Manzanilla et al. 2006), hence only minor amount of unprotected butyrate can proceed to the small intestines.

Butyrate, when applied as protected-form feed additive can reach the small or even the large intestinal tract, where higher pH (~pH 5-8) is characteristic, therefore, it is found mostly in its dissociated form when released (Sellin 1999). This is equally true for the butyrate derived from local (microbial) SCFA synthesis, therefore, as passive transport is impossible in this state, active transport is the principal way of absorption. This energy consuming transport mechanism

is so effective, that only 5% of the total SCFAs avoids absorption and is excreted with the feces (den Besten et al. 2013).

Three transport protein types are involved in the above mentioned active transport process. The first type is SCFA/HCO_3^- exchanger (Mascolo et al. 1991; Harig et al. 1996), which is discovered but not fully characterized yet. Another type of transporters is the member of the family of monocarboxylate transporters (MCTs), importing SCFA anions coupled with a H^+, decreasing intracellular pH (Hadjiagapiou et al. 2000). The third type belongs to the group of sodium-dependent monocarboxylate transporters (SMCTs), performing SCFA anion and Na^+ cotransport (Takebe et al. 2005; Teramae et al. 2010). These types of transporters are located on the apical and basolateral membranes of the enterocytes, respectively, being responsible for both the active absorption of SCFAs into the cytoplasm and for the forward of the non-metabolized butyrate from the enterocyte to the portal circulation.

Butyrate proved to exert its extensive and manifold beneficial effects already in the intestinal lumen, where both orally applied and microbially produced butyrate reach the highest concentration in the body, acting on the resident microflora and the host organism at several points.

2.3. Action of butyrate on cellular level

2.3.1. Epigenetic regulator

The eukaryotic chromosome is the highest level of deoxyribonucleic acid (DNA) organization that is composed of condensed chromatin, built up by nucleosomes (Arents et al. 1991). Nucleosomes are a bit less than two turns of DNA segments wound around histone octamers formed by a set of eight globular histone core proteins (H2A, H2B, H3, H4; Kouzarides, 2007). This formation is stabilized by the electrostatic attraction between the negatively charged phosphate groups of the DNA and the positively charged amino acids of the N-terminal tails of the histone proteins. The compactness of the chromatin structure and thus the intensity of gene expression partly depend on the strength of this stabilizing force. Therefore, any impact weakening the electrostatic interaction results in the transcriptionally more active euchromatin (loose) form, enhancing the accessibility of genes to the transcription factors, and vice versa, the compact heterochromatin form with strong interactions prevents gene expression (Bernstein et al. 2007). Acetylation and ubiquitination of lysine residues, phosphorylation of serines, methylation of lysine and arginine and sumoylation of the N-terminal tail of histone proteins hide their positive charge, altering chromatin structure to the transcriptionally more active relaxed form (Berger 2007). In the process of acetylation, acetyl functional groups are attached to the lysine residues protruding from the histone core of the nucleosome by histone acetyl transferase (HAT) enzyme, while histone deacetylase (HDAC)

enzyme catalyzes the opposite process (Rada-Iglesias et al. 2007). The balance of the activity of these two enzymes determine the degree of acetylation, hence the acetylation balance of the histone proteins.

Butyrate, if not consumed in metabolic processes, acts as an epigenetically active molecule, being able to inhibit HDAC, causing hyperacetylation of histones and consequently the possibility of intensified gene expression *in vitro* in cultured cells (Candido et al. 1978; Roediger 1982) and *in vivo* in the cecum of piglets (Kien et al. 2008) and in the hepatocytes of chicken (Mátis et al. 2013a, b). Further, butyrate proved to affect micro ribonucleic acid expression (miRNA: small non-coding ribonucleic acid (RNA) segments; Esquela-Kerscher and Slack 2006) through histone acetylation in bovine (Li et al. 2010) and also in human colorectal cancer cells (Bishop et al. 2017). The miRNA binds to the 3'-untranslated region on the given messenger ribonucleic acid (mRNA), suppressing its expression or targeting the mRNA for degradation (Esquela-Kerscher and Slack 2006). Regarding that miRNAs are involved in the regulation of the transcription of c.a. 30% of all protein-encoding genes in human, almost all metabolic pathways might be altered by epigenetically active factors affecting miRNA expression (Esquela-Kerscher and Slack 2006; Mathers et al. 2010).

DNA methylation also modifies the transcriptional pattern of certain genes by linking methyl groups primarily to those 5' cytosine residues that are followed by a guanine residue on the promoter region of the gene, leading to the spatial inhibition of the binding of transcriptional factors and thus causing gene silencing (Patel et al. 2005; McKay and Mathers 2011). This process is catalyzed by DNA methyltransferases using S-adenosylmethionine as methyl donor, and is assumed tissue-specific, at least in human (Ollikainen et al. 2010). Butyrate was reported to perform hypermethylation of DNA, therefore it has the capacity to regulate gene expression this third way as well (Cho et al. 2009).

2.3.2. Receptor mediated action

The intensity of metabolic processes is determined by the activity of certain enzymes, that can be regulated through intracellular signaling pathways. The first step of these pathways is always the binding of a molecule (ligand) to a receptor on the extracellular side of the cell membrane. The largest and the most diverse family of transmembrane proteins is called G-protein-coupled receptors (GPCRs) with characteristic seven membrane-spanning α-helical segments (Rosenbaum et al. 2009), mediating the most of the cellular responses to hormones, neurotransmitters and environmental stimulants. Binding of a ligand to the extracellular surface of a GPCR leads to conformational changes that promote the interaction of the intracellular surface of the receptor with distinct classes of G-protein heterotrimers (Strader et al. 1994). G-protein is composed of three subunits (α, β and γ) on the cytoplasmic surface of the plasma membrane (Conklin and Bourne 1993; Neer 1995), of which α subunit binds guanosine-

diphosphate in inactive state. The activated GPCR triggers the exchange of guanosine-diphosphate to guanosine-triphosphate, leading to the dissociation of the G-protein from the receptor and α subunit from the βγ complex, thus the activation of both (free α subunit and the βγ complex) parts of G-protein (Conklin and Bourne 1993; Neer 1995). These activated parts are then able to interact with distinct compounds, to activate them and induce intracellular signaling processes primarily through cyclic adenosine monophosphate (cAMP) and phosphoinosytol pathways.

The SCFA-triggered G-protein receptor 43 (GPR43) can be found in white and brown adipose tissue, pancreas and in the large intestines with the highest expression rate in immune cells (Regard et al. 2008), ensuring the possibility for SCFAs to be involved in leukocyte activation (Vinolo et al. 2011; Wang et al. 2015). The other SCFA sensitive G-protein receptor 41 (GPR41) has an even wider distribution compared to GPR43, detected in adipose tissues, pancreas, spleen, lymph nodes, bone marrow and mononuclear cells (Byrne et al. 2015), but the most important sites are the intestines and the nervous system, where butyrate directly modulates sympathetic nervous system activity to maintain metabolic homeostasis and regulate body energy expenditure through GPR41 (Kimura et al. 2011). Another major GPCR activated by butyrate is G-protein receptor 109A (GPR109A; Thangaraju et al. 2009), found on the surface of small intestinal epithelial cells, colonocytes and immune cells. In this case, butyrate exerts its anti-inflammatory properties by the inhibition of the production of cytokines and proinflammatory enzymes (Fu et al. 2015) and anti-tumor effect by inducing apoptosis of cancer cells (Thangaraju et al. 2009). On the other hand, GPR109A signaling activates the inflammasome pathway leading to the differentiation of certain T-cells (Singh et al. 2014) and increases the enteral interleukin-18 secretion (Macia et al. 2015).

2.4. Role of butyrate in the gastrointestinal tract

2.4.1. Energy production in the enterocytes

SCFAs, but primarily butyrate absorbed by enterocytes can be released into the portal circulation on the basolateral side of the cells; however, most of it is utilized in different metabolic pathways, especially as energy source in catabolic processes (Roediger 1982; Guilloteau et al. 2010). Colonocytes prefer butyrate rather than other SCFA molecules for energy production: up to 60-70% of total energy requirement of enterocytes can be derived from butyrate in this section (Roediger 1982). In energy producing catabolic pathways butyrate is first oxidized into two acetyl~CoA via β-oxidation, which can enter the citrate cycle to be oxidized to CO_2, while reduced hydrogen carrier molecules transport hydrogen to the members of the respiratory chain for adenosine triphosphate (ATP) production. Under certain conditions, the acetyl~CoA molecules are preferably converted to ketone bodies, involved in the

cholesterol synthesis, energy production or engaged in the synthesis of lipids as precursor (Guilloteau et al. 2010).

2.4.2. Regulatory role in the gastrointestinal epithelium

Intestines is the organ with the highest exposure to butyrate, therefore, the most diverse effects are observable in this area.

Another reasons for butyrate's growth promoting and feed conversion ratio improving effect are the histomorphological changes it induces: augmented villus height (Pelicano et al. 2005; Adil et al. 2010) and deeper crypts (Antongiovanni et al. 2007) were reported with concomitant butyrate application, resulting in more efficient nutrient absorption (Moquet et al. 2018b). This phenomenon can be explained by the fact that butyrate enhances the expression of early response genes, regulating cell division, differentiation, growth and apoptosis (Maclean et al. 1998), as well as stimulates proglucagon expression, a precursor protein of glucagon hormone which is responsible for the cellular proliferation in the gut in human (Woodard and Tappenden 2008), and *in vitro* in rat (Zhou et al. 2006).

The diverse and dense colonizing microflora claims a relatively hyposensitive intestinal immune system (Chang 2014), which at the same time must ensure adequate protection for the organism. Butyrate enhances colonic mucin, antimicrobial peptide and T-cell production (van Immerseel et al. 2010; Arpaia et al. 2013), and modulates the function of macrophages (Chang et al. 2014), as well as increases IgY natural antibody level in the duodenum and jejunum (Moquet 2018a). Further, due to its epigenetic action, butyrate proved to increase the expression of tight junction proteins, thus strengthens the barrier function of the intestinal epithelium (Wang et al. 2012).

Intestinal epithelium is not a physical barrier only, but also functions as a very important first line metabolic barrier for orally ingested xenobiotics (Obach et al. 2001), influencing their bioavailability and toxicity (Le Poul et al. 2003). Butyrate, if not metabolized in the cell, can act through the above mentioned epigenetic and receptor mediated pathways, modulating the expression of several inducible enzymes, amongst others, certain cytochrome P450 enzymes (CYPs), that can be triggered by dietary factors (Kulcsár et al. 2017).

Butyrate proved to decrease the incidence of inflammatory bowel disease and colorectal cancer (Scheppach and Weiler 2004; Wong et al. 2006; Singh et al. 2014) via the reinforcement of the apoptotic processes (Thangaraju et al. 2009).

In both mammalian and avian species, pancreatic insulin secretion is primarily controlled by incretin hormones, such as glucose-dependent insulinotropic polypeptide or gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP-1). GIP is ejected by the small intestinal K-cells, with the highest density in the duodenum (Mortensen et al. 2003). GLP-1 is a product of the glucagon gene (Mojsov et al. 1986), released by the L-cells in the small and

large intestines as well (Baggio and Drucker 2007; Doyle and Egan 2007). These key members of the enteroinsular axis (Creutzfeldt 1992) enhance the glucose-dependent insulin secretion of pancreatic β-cells, however, in chicken, GLP-1 exerts its insulinotropic action more likely by increasing the somatostatin production of the δ cells of the pancreatic islets rather than by direct stimulation of β-cells (Watanabe et al. 2014). Further, GLP-1 exerts its glucoregulatory pancreatic action by the glucose-dependent inhibition of glucagon production as well (Baggio and Drucker 2007). Recent investigations have justified that orally applied butyrate increases pancreatic insulin secretion by inducing elevated plasma concentrations of GLP-1 and GIP in mice (Lin et al. 2012), and the effect of butyrate bolus exposure on plasma GIP concentrations was also detected in chicken and rabbit (Mátis et al. 2018).

2.4.3. Effects on the intestinal microbiome

2.4.3.1. Characterization of the intestinal microbiome in chicken

The gastrointestinal tract of poultry is shorter relative to body length and characterized by a shorter digesta transit time, compared to mammals (Pan and Yu 2014). Nevertheless, avian ceca forming a pair of blind pouches have slower transit time and are ideal for a diverse microbiome with notable active microbe-microbe, microbe-host and microbe-diet interactions, thus having a huge impact on host nutrition and health.

The poultry intestine harbors *Firmicutes*, *Bacteroidetes* and *Proteobacteria* the most, but *Clostridium*, *Lactobacillus*, *Bifidobacterium*, *Ruminococcus* and *Bacteroides* species also occur (Wei et al. 2013). The exact composition of the microbiome depends on several factors, including diet composition and structure, ingesta transit time, rearing environment and the age of birds (Amit-Romach et al. 2004; Pan and Yu 2014). For instance, rye-, barley- or wheat-based diets with high NSP level favor the proliferation of *Clostridium perfringens* and predispose young chicks to necrotic enteritis (Choct et al. 1996; Jia et al. 2009). As an opposite, xylanase and glucanase enzyme supplementation of the diet proved to increase the ratio of lactic acid producers and decrease the number of adverse bacteria or pathogens, e.g. *Escherichia coli* (Rodríguez et al. 2012).

After the digestion and absorption of most readily digestible carbohydrates in the proximal section of the gastrointestinal tract, residual digestible carbohydrates and carbohydrates indigestible by the host are degraded to their compositional sugar molecules by anaerobic microbial fermentation, serving as precursors for SCFA synthesis. Such fermentation is detectable from crop to the cecum in chickens, but is primarily characteristic in the cecum, where an increasing amount of SCFAs is produced after hatch which peaks in 15 day old animals and remains stable afterwards (van der Wielen et al. 2000), playing an essential role in the intestinal development. Gut microbiota also takes part in the avian nitrogen metabolism

by catalyzing the conversion of uric acid to ammonia, derived from the urine, delivered by the retrograde peristalsis from the cloaca (Braun and Campbell 1989). The ammonia is then absorbed by the host and used for the synthesis of certain amino acids (e.g. glutamine; Vispo and Karasov 1997), or incorporated into the bacterial cellular proteins, also serving as a possible amino acid source for the birds (Metges 2000).

The avian intestines might provide an ideal niche not only for beneficial, but also for bacteria with possible risk to human health, such as *Escherichia coli*, *Salmonella* or *Campylobacter* species. Amongst the numerous representatives of the *Campylobacter* genus, *Campylobacter jejuni (C. jejuni)* is the predominant species colonizing commercial broiler flocks and thus has the highest impact from human infection point of view (Heuer et al. 2001; Wittwer et al. 2005).

2.4.3.2. Selective antimicrobial effect of butyrate

SCFAs, and especially butyrate possess selective antimicrobial property, inhibiting the growth of numerous pathogenic bacteria, e.g. enterotoxic *Escherichia coli* and *Campylobacter jejuni* strains, *Clostridium* and *Salmonella* spp. (Fernández-Rubio et al. 2009), while being harmless or even beneficial to the members of eubiotic organisms, thus stabilizes the intestinal microflora (Hu and Guo 2007).

This selective antimicrobial effect on most enteral pathogens is traditionally explained by the ability of the undissociated butyric acid molecule to pass across the bacterial cell membrane and to dissociate in the more alkaline interior milieu (Kashket 1987). After dissociation, the ionised, anionic form cannot be transported further through the semipermeable membrane by passive diffusion, therefore, it is being captured in the bacterial cell, while released protons acidify the cytoplasm. Since most enteral pathogens are outstandingly sensitive to declined intracellular pH, increased intensity of active pumping out of the accumulated protons results in cellular ATP depletion. On the other hand, augmented cytoplasmic proton concentration can increase the sodium import as well by enhancing the Na^+/H^+ antiport mechanism, elevating the turgor of the cell. Third, accumulated butyrate as an epigenetically active molecule may also influence bacterial gene expression, for instance in *Salmonella* spp., where butyrate declined the expression of Salmonella pathogenicity island gene, responsible for colonization and virulence of the bacteria (Gantois et al. 2006), causing reduced invasiveness of microbes in intestinal epithelial cells *in vitro* (van Immerseel et al. 2003). However, regarding that the pH of the proximal part of the large intestines is close to the pKa of butyrate (~pH 5-6 vs. pKa 4.82), the proportion of the undissociated, thus effective form of butyrate against pathogenic bacteria strongly depends on the pH of the luminal content, being higher in more acidic milieu.

However, most fermentative bacteria (such as *Lactobacillus* spp. and *Streptococcus bovis*), being part of the symbiotic enteral microflora, are less sensitive to the declined

intracellular pH. As the pH gradient across the cell membrane of these bacteria remains lower, they are particularly protected from anion accumulation (Gálfi and Neogrády 1996). In addition, such bacteria can utilize butyrate as a probiotic energy source as well (Candela et al. 2010), mediated by increased cellular acetyl~CoA production, which can enter the citrate cycle or can be used for replenishing intermediates of the citrate cycle via the glyoxylate shunt.

2.4.3.3. General properties and colonization dynamics of Campylobacter jejuni

C. jejuni is a microaerophilic, thermotolerant, curved Gram-negative rod (1.5-5 µm) with cork-screw motility and cytochrome oxidase positivity, primarily associated to poultry (Levin 2007; EFSA 2021). Regardless of the rearing technology, this bacterium is rarely detected in birds younger than two weeks (Conlan et al. 2007; Allen et al. 2011; Patriarchi et al. 2011), suggesting certain maternal immunity this age (Cawthraw and Newell 2010). Vertical transmission in the hatchery can be considered insignificant, *Campylobacter* colonization is due to old litter, insects, other (farm) animals, vehicles and farm workers (Newell and Fearnley 2003; Callicott et al. 2006). The chickens are infected via the fecal-oral route naturally and the organism establishes itself in the small and large intestines, with the highest number in the ceca, colon and cloaca (Beery et al. 1988; Sahin et al. 2015; Levin 2007), and to a lesser extent in the liver, spleen, muscles, thymus and bursa of Fabricius (Cox et al. 2005). *Campylobacter* spp. colonize in the gastrointestinal tract by many strategies including rapid replication in the mucus layer and temporary invasion into the intestinal epithelium (van Deun et al. 2008b). Molnár et al. (2015) found altered colonization dynamics when feeding wheat-based diet to broilers, but age, genotype and immune response of birds, as well as bacterial strain also influence gastrointestinal colonization (Pielsticker et al. 2012; Humphrey et al. 2014; Han et al. 2016). The assignment of *C. jejuni* is commensal rather than pathogenic, but recent investigations suggest that *Campylobacter* colonization might be negatively associated with chicken intestinal function, growth rate and welfare (Williams et al. 2013; Awad et al. 2015). The infection of one chicken results in the persistent high number of *Campylobacter* present in the gut, then the vast majority of the animals become colonized within a few days that might affect up to 100% of the flock at slaughter (Newell and Fearnley 2003; Sahin et al. 2015).

2.4.3.4. Campylobacter jejuni as risk for human health

Campylobacteriosis, especially *C. jejuni* infection is the most frequent source of gastrointestinal infections in industrialized countries with a higher incidence than *Salmonella* infections (Bereswill and Kist 2003; Wigley 2015; EFSA 2021). Even low number (2-3 cells per ml; Robinson 1981) of *C. jejuni* causes severe zoonotic, foodborne diseases when transmitted with contaminated products of poultry origin, typically with raw broiler meat with fecal contamination at the slaughter process (Gölz et al. 2014). The infection is generally mild, but

can be fatal among very young children, aged or immunodeficient individuals. The most frequent clinical symptoms of infection include diarrhea (often bloody), abdominal pain, headache, fever, nausea or vomiting (Ternhag et al. 2007), but in case of bacteremia multi-organ inflammation, miscarriage, reactive arthritis and Guillain-Barré syndrome has also been reported in some cases (Blaser et al. 1979 and 1986). The symptoms last for 3 to 6 days.

The worldwide high incidence and spread of *Campylobacter* infections, its duration and possible complications underline its enormous importance from a socio-economic perspective. Therefore, several pre- and post-harvest intervention methods have already been developed and improved in order to diminish *Campylobacter* contamination of poultry meat, with partial success.

Since carcass contamination is directly proportional with *C. jejuni* counts of the ingesta, bacterial colonization is intended to be controlled already in the live phase (Ghareeb et al. 2013; EFSA 2021). Due to food safety regulations, the short rearing period of broilers and the great antigenic variability of different *Campylobacter* strains, application of antibiotics or vaccines are not possible ways of *Campylobacter* eradication, but – amongst others – biosecurity restrictions, acidification of drinking water and litter, pre- and probiotics (latter also called competitive exclusion treatment e.g. with *Escherichia coli* and *Klebsiella pneumoniae*) as well as administration of bacteriophages, bacteriocins or organic acids as feed additives are all parts of the intervention strategies, and *in ovo* vaccination might be another promising method in the future (Skanseng et al. 2010; Hermans et al. 2011; Ghareeb et al. 2013; Allain et al. 2014). As post-harvest methods, freezing, hot water treatment, irradiation and chemical decontamination can be mentioned, although the latter is not allowed in the European Union (Wagenaar et al. 2006; Berrang et al. 2007; Cox and Pavic 2010; EFSA 2011).

Considering that these efforts seem to be not successful enough in reducing the prevalence of foodborne campylobacteriosis (EFSA 2021), some alternative intervention strategies have to be introduced, such as the application of novel feed additives, which can be promising tools to combat bacterial gut infections (van Immerseel et al. 2003). Contrary to the presence of the commensal *C. jejuni* or other *Campylobacter* species that does not require antimicrobial treatment of the chicken, fluoroquinolones and macrolides are the most frequent and best choices in human cases (Allos 2001). Thus, the increasing – although strain-specific – antibiotic resistance of *Campylobacter* spp., found especially in conventional poultry farms is a major concern for public health (Endtz et al. 1991; Nelson et al. 2007).

2.5. The extraintestinal role of butyrate

2.5.1. General metabolic effects

That amount of butyrate which is absorbed but not processed by the enterocytes reaches the liver via the portal veins directly, therefore, liver is the organ with the highest exposure to butyrate beyond the gut. Hepatocytes can use the butyrate to gain energy through β-oxidation or it can serve as precursor for fatty acid, cholesterol or under certain circumstances, ketone body synthesis (den Besten et al. 2013).

Hepatocytes have a central role in the detoxification of xenobiotics, which is partly performed by inducible microsomal CYP enzymes. The gene expression of some CYP subfamilies can be modulated by butyrate delivered from the intestines, presumably through epigenetic or receptor mediated pathways (Mátis et al. 2013a, b; Csikó et al. 2014; Kulcsár et al. 2017).

Liver is the main regulator of the carbohydrate and lipid metabolism as well and one of the main targets of butyrate. When butyrate binds to GPR41 or GPR43 receptors on the surface of hepatocytes, adenosine-monophosphate activated protein kinase (AMPK) is getting activated by phosphorylation, promoting the gene expression of peroxisome proliferator activated receptor alpha (PPARα) participating in the β-oxidation of fatty acids, lipogenesis, gluconeogenesis and glycogenesis (Brown et al. 2003; Canfora et al. 2015). As a result, intravenously administered butyrate proved to decrease lipogenesis and concomitantly increase glucose tolerance (den Besten 2013). Additionally, butyrate has the capacity to modify the insulin receptor β (IRβ) expression of the hepatocytes diversely depending on the origin and the way of application (Mátis et al. 2015; Kulcsár et al. 2016).

Butyrate, which is not processed in the liver is forwarded into the systemic circulation, by which – although its concentration does not reach that as measured in the portal veins (Egorin et al. 1999; Knudsen et al. 2005) – a detectable amount of butyrate is able to reach the extrahepatic tissues and exert its effects there (Gao et al. 2009; Kulcsár et al. 2016).

In the brown adipose tissue, a thermogenic effect of butyrate was observed due to the activation of uncoupling protein 1 expression in mice (Gao et al. 2009). Further, butyrate has the potential to modulate inflammatory processes in the adipose tissues by the reduction of proinflammatory cytokine and chemokine production (Canfora et al. 2015), as well as to influence the insulin sensitivity of this tissue (Mátis et al. 2015; Kulcsár et al. 2016).

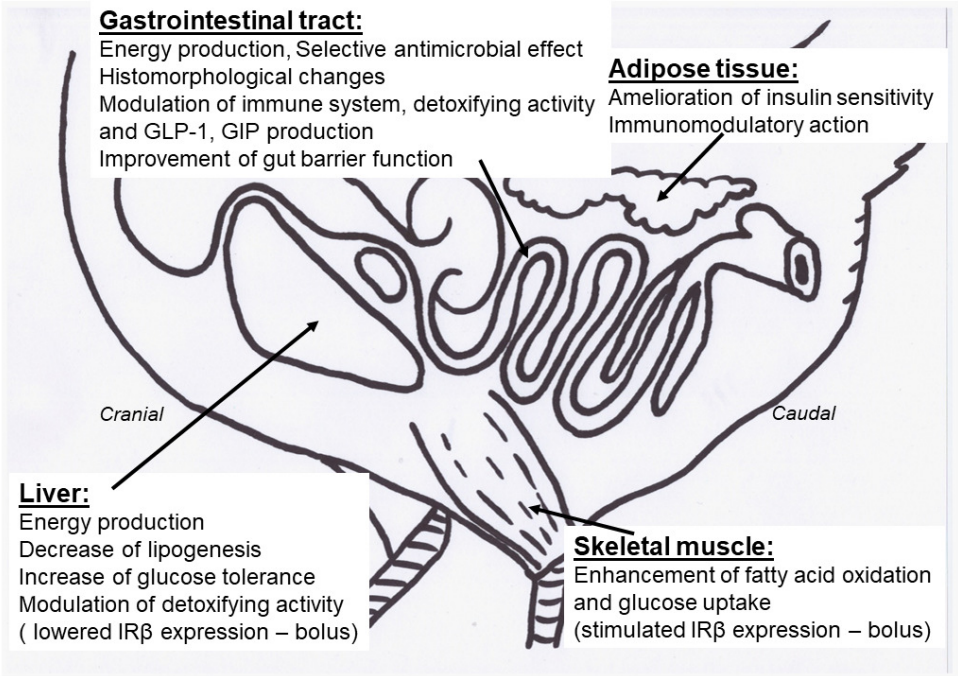
In the skeletal muscles AMPK mechanism leads to the amelioration of fatty acid oxidation and glucose uptake of the cells using glucose transporter 4 (GLUT4) in mammals as well as increases glycogenesis, and consequently improves insulin sensitivity (Canfora et al. 2015). Further, daily intraingluvial butyrate bolus treatment was found to upregulate IRβ expression in

the gastrocnemic muscle of chickens but had an opposite effect in the liver (Mátis et al. 2015), suggesting the ability of this molecule to act on glucose shifting amongst tissues.

Due to its manifold biological actions, butyrate is able to improve the growth performance of broilers, characterized by significantly better body weight gain and feed conversion ratio results (Hu and Guo, 2007). Literature data indicate that butyric acid glycerides increased the carcass weight and relative breast meat yield of broiler chickens, with more pronounced effects under suboptimal circumstances, namely after infection with *Eimeria* oocysts (Leeson et al. 2005). Accordingly, butyrate supplemented feed proved to increase carcass yield of broilers and had an inverse effect on abdominal fat depots (Panda et al. 2009), while Antongiovanni et al. (2007) found no impact of butyric acid glycerides on the carcass composition of chickens. However, there is still lack of further knowledge on how different application forms of butyrate may affect the chemical composition of chicken meat and thus influence meat quality.

The main actions of butyrate are summarized in **Figure 1**.

Figure 1. Overview of the main gastrointestinal and extraintestinal actions of butyrate in birds



GLP-1: Glucagon-like peptide 1; GIP: Glucose-dependent insulinotropic polypeptide; IRβ: Insulin receptor β subunit. The figure was created by the author.

2.6. The avian carbohydrate metabolism

Carbohydrate metabolism of birds is characterized by 1.5-2.0-fold higher blood sugar concentration than observed in mammals of similar body mass (Braun and Sweazea 2008), partly due to the relative insulin resistance of the extrahepatic tissues, compared to mammals (Dupont et al. 2004). Decreased insulin sensitivity of primarily the skeletal muscles and adipose

tissue ensures the physiologically higher blood sugar level, regardless of feed restriction, fasting, migration or changes in photoperiod (Braun and Sweazea 2008; Scanes and Braun 2013). The functional relevance of the physiological hyperglycemia is not fully elucidated yet, explained traditionally by the need for energy during flying lifestyle (Clarke and Portner 2010). In contrast, all bird species exhibit high blood glucose level (with interspecific variance), and only the energy requirement of take-off and short-haul flights are covered by glucose oxidation (Butler 2016).

2.6.1. The glucose uptake and carbohydrate homeostasis of birds

Similar to what is observed in mammalian species, the glucose uptake of the cells needs several types of glucose transporter proteins. One group of transporters is glucose transporter transmembrane proteins (GLUTs), performing facilitated passive transport across the cell membrane (Braun and Sweazea 2008). Other main types of transporters belong to the group of sodium-glucose co-transporters (SGLTs), featuring secondary active transport in the glucose turnover. In birds, the most important member of this group is SGLT-1, expressed on the apical side of the enterocytes (Braun and Sweazea 2008). SGLT-1 together with GLUT-2 is responsible for the absorption of glucose from the ingesta, but to a lesser extent, paracellular diffusion also takes place both in mammals and birds (Karasov and Cork 1994). SGLT-1 carries one glucose molecule coupled with two sodium ions into the intracellular space. SGLT-1 has been confirmed in chicken small and large intestines (Garriga et al. 1999). The driving force of this transport is the lower intracellular electrochemical gradient of sodium, ensured by the transmembrane sodium-potassium adenosine triphosphatase (or sodium-potassium pump), localized in the basolateral membrane of the enterocytes and maintaining active sodium-potassium antiport mechanism. Approximately 70% of the absorbed glucose is forwarded into the portal capillaries on the same basolateral side of the cell by GLUT-2, while the rest is converted into lactic acid in the anaerobic glycolysis intracellularly and is released into the circulation in this form (Braun and Sweazea 2008). In the followings, the glucose transport of the liver and skeletal muscles will be detailed only, which are the most relevant organs from the PhD study point of view.

Portal veins carry glucose directly to the liver, which is – like in mammals – the central organ in the regulation of blood glucose level in most bird species, maintaining the physiological high blood glucose level by the aid of gluconeogenesis and glycogenolysis. Thus, carbohydrate-free diet or starvation of birds manifests in reduced hepatic glycogen content, meanwhile blood glucose level remains unchanged (Tinker et al. 1986; Braun and Sweazea 2008). In the liver, GLUT-1, the partly insulin-dependent GLUT-2 and GLUT-3, as well as GLUT-8 proteins have been confirmed, with a predominant role of GLUT-2.

The skeletal muscle consumes a notable amount of glucose, and also plays a key role in the avian glucose homeostasis. The glucose uptake of skeletal muscle cells is mostly implemented with GLUTs, both in birds and mammals. However, the insulin dependent, dominant GLUT-4 transporter is not detectable in birds (Seki et al. 2003; Dupont et al. 2004). In chicken, its role is assumed to be partially fulfilled by the also insulin sensitive GLUT-12 (Coudert et al. 2015). Despite this, insulin-evoked glucose uptake of muscle cells is insignificant, due to the high expression rate of the insulin-independent GLUT-1 and GLUT-3 transporters (Kono et al. 2005).

In the muscle cells, glucose can enter the glycogenesis, be used for energy production, or involved in the amino acid production, essential for the large-scale muscle protein synthesis in the phase of intensive growth (Braun and Sweazea 2008). Further, the glycogen stores of muscles significantly contribute to the normalization of high blood glucose level in case of starvation (Tinker et al. 1986), but have only little importance under physical stress, such as extended flights (Schwilch et al. 1996; Jenni-Eiermann et al. 2002).

The summary and comparison of the mammalian and avian glucose transporters in different organs are presented in **Table 1.**

Table 1. Overview of the expression pattern of mammalian and avian glucose transporters

Glucose transporter	Mammals	Insulin dependency in mammals	Birds	Insulin dependency in birds
GLUT1	brain, erythrocyte	no	brain, cardiac muscle, adipose tissue, kidney, *corpus gelatinosum*	no
GLUT2	liver, kidney, enterocyte	no	liver, kidney, enterocyte	**partly**
GLUT3	brain, placenta	no	brain, cardiac muscle, kidney, adipose tissue, erythrocyte	**partly**
GLUT4	cardiac and skeletal muscle, adipose tissue	**yes**	*does not exist*	-
GLUT8	adipose tissue	no	cardiac muscle, adipose tissue	no
GLUT12	cardiac and skeletal muscle	**yes**	cardiac and skeletal muscle	**yes**

GLUT: Glucose transporter.

Note: adapted partly from Braun and Sweazea, 2008, Sturkie's Avian Physiology, 2014.

2.6.2. The endocrine regulation of carbohydrate metabolism

The avian carbohydrate homeostasis is regulated by a variety of hormones, of which the most important members are secreted in the pancreas, containing α, β, δ and F-cells in the Langerhans islets, where glucagon (α), insulin (β), somatostatin (δ) and avian pancreatic polypeptide (F-cells) are produced (Hazelwood 1973; Sitbon and Mialhe 1980). The two most

important hormones are the plasma glucose lowering insulin and its antagonist glucagon in 6-19-fold higher concentration than insulin (Ruffier et al. 1998).

Most studies conducted on birds have revealed little or no effect of insulin on the plasma glucose level and the glucose uptake of distinct tissues (Tokushima et al. 2005; Sweazea et al. 2006). The fact that the plasma insulin concentration is c.a. one tenth of that of rat is in line with the observation that the number of β cells and the insulin content of the pancreas is far lower in birds (Hazelwood 1973; Dupont et al. 2004). Unlike in mammals, pancreatic insulin production and release is quite irresponsive to glucose stimulus (Hazelwood 1973). However, administration of tolbutamide is one of the few methods to increase plasma insulin concentration and causes transient hypoglycemia in birds fed standard diet (Seki et al. 2001), and also in mammals (Proks et al. 2002), suggesting that the mechanism of pancreatic insulin delivery shall be similar in these species (Danby et al. 1982; Tinker et al. 1986).

The concentration of the antagonistic glucagon in the tissue of chicken pancreas is c.a. 8-10-fold higher than in mammals (per unit weight; Hazelwood 1973), and unlike insulin, has a very pronounced effect on the avian tissues, responding with elevated blood glucose, triglyceride, as well as free fatty acid and glycerol concentrations. Similar to mammals, its function is to augment lowered blood glucose level by the stimulation of glycogenolysis and gluconeogenesis in the liver (Hazelwood 1973), therefore, exogenous glucose stimulus proved to moderate glucagon release from the pancreatic α cells of birds, independent of the paracrine effect of the insulin (Ruffier et al. 1998).

The GIP and GLP-1 also take an essential place in the regulation of glucose metabolism through the mediation of the pancreatic hormone release in birds (Litwack 2010). In general, both incretins are recognized by their surface receptors expressed by the β cells, inducing proliferation, increased resistance to apoptosis and insulin production (Baggio and Drucker 2007). Nevertheless, GLP-1 receptors were detected on the surface of islet somatostatin producing δ cells and not β cells in chicken, suggesting that GLP-1 might stimulate insulin secretion via a signaling different from that of mammals (Watanabe et al. 2014).

The overview of the carbohydrate homeostasis-regulating avian hormones is presented in **Table 2.**

Table 2. Main characteristics of hormones regulating glucose homeostasis

Hormone	Place of production	Place of action	Action
Insulin	pancreatic β-cells	distinct tissues	stimulation of glucose uptake
Glucagon	pancreatic α-cells	liver	enhancement of glygogenolysis and gluconeogenesis
Somatostatin	pancreatic δ-cells	pancreas	inhibition of glucagon and insulin secretion
Avian pancreatic polypeptide	pancreatic F-cells	pancreas liver adipose tissue	suppression of insulin release stimulation of glycogenolysis enhancement of lipolysis
GIP	intestinal K-cells	pancreas	stimulation of insulin release
GLP-1	intestinal L-cells	pancreas	stimulation of insulin release
Glucocorticoids	*zona fasciculata* of adrenal cortex	liver	stimulation of gluconeogenesis
Adrenalin	adrenal medulla	liver, skeletal muscle	enhancement of glycogenolysis
Growth hormone	pituitary gland	liver adipose tissue	inhibition of gluconeogenesis from amino acids promotion of lipolysis

GIP: Glucose-dependent insulinotropic polypeptide; GLP: Glucagon-like peptide 1.

Note: adapted partly from Sturkie's Avian Physiology, 2014.

2.6.3. Insulin homeostasis and the insulin signaling pathway

Despite the reduced physiological insulin sensitivity of the avian extrahepatic tissues compared to mammals, insulin is one of the most important regulators of growth, carbohydrate, lipid and protein metabolism of chicken (Józefiak et al. 2010; Scanes and Braun 2013), also ameliorating feed utilization efficiency (Duchène et al. 2008a, b). Amongst others, its production is determined by genetic background, nutrition and age of the animal (Józefiak et al. 2010).

Insulin exerts its multiple effects through a complex intracellular signaling mechanism, which is well known in details in mammalian species, and starts with the reception of the hormone on the surface of distinct cells.

Once insulin binds to the insulin receptor α subunit (IRα), the conformational change leads to the autophosphorylation of the β subunit due to its tyrosine kinase activity (IRβ; White and Kahn 1994). IRβ phosphorylates insulin receptor substrate 1 (IRS-1), activating mitogen-activated protein-kinase (MAP-kinase) cascade and phosphatidylinositol-3-kinase enzyme (PI3K), inducing elevated concentration of phosphatidylinositol trisphosphate (PIP_3; White and Kahn 1994). This member of the cascade participates in the activation of protein kinase B (PKB or Akt) and protein kinase C (PKC). Activation of PKB inactivates glycogen synthase kinase 3 (Welsh and Proud 1993), resulting in the storage of glucose in form of glycogen (Lawrence and Roach 1997), but glycolysis and lipogenesis are enhanced as well (White and Kahn, 1994). Further, mammalian (also known as mechanistic) target of rapamycin (in the followings, referred as mammalian target of rapamycin or mTOR) is also activated by PKB, known as the cardinal stimulator of glycolysis, lipogenesis, and muscle growth (White and Kahn 1994; Dupont

et al. 2009 and 2012). PKC stimulates GLUT-4 mediated glucose uptake of the cells, being involved in the translocation of GLUT-4 containing vesicles to the intracellular surface of the plasma membrane in mammals (Uldry and Thorens 2004). The lack of GLUT-4 proteins makes the function of PKC questionable in birds, and it has not been clarified yet whether it participates in the mediation of the also insulin-dependent GLUT-12 transporters, expressed in the skeletal myocytes of chicken (Coudert et al. 2015).

Regarding structural and functional properties, insulin receptors (IRs) of chicken are similar to the mammalian ones (Dupont et al. 2009), however, certain important differences occur. Compared to rat, although the amount of IR receptors in chicken skeletal muscles is comparable, IRS-1 and PI3K proteins are of notably higher number in chicken (Dupont et al. 2004), and adipose tissues express significantly lower level of IR and IRS-1 proteins under physiological circumstances in this species (Dupont et al. 2012). Further, the basal phosphorylation degree of IRβ subunit in chicken skeletal myocytes is double, however, there is no significant difference when the extent of tyrosine residue phosphorylation of IRS-1 is compared (Dupont et al. 2004). Additional difference is that in the skeletal muscle, the physiological activity of PI3K is c.a. thirtyfold higher in chicken (Dupont et al. 2004). Physiologically elevated phosphorylation rate of IRβ and increased basal PI3K activity in chicken skeletal muscle might indicate that the insulin signaling cascade can be regarded as already activated, independent of insulin stimulus. This activated system cannot be triggered by the physiological plasma concentration of insulin, therefore, this phenomenon could be a possible explanation of the relative insulin refractoriness of avian skeletal muscle (Dupont et al. 2004). In line with this finding, the phosphorylation rate of IRβ and IRS-1 is not affected by fasting in the skeletal muscles and adipose tissues of chicken, but decreases remarkably in the hepatocytes of chicken and in all the tissues of rats after fasting (Dupont et al. 2009 and 2012). Additionally, PI3K activity is altered in the liver of chicken by changes in plasma insulin concentrations, but remains unaffected in the muscle cells (Dupont et al. 2009).

In spite of the relative insulin refractoriness of avian tissues, the activation of insulin signaling is very important, as insulin is one of the main factors involved in the modulation of protein synthesis and muscle growth in chicken (Duchène et al.2008a, b; Józefiak et al. 2010).

As a downstream element of the intracellular insulin signaling, mTOR manifests its muscle growth stimulating effect by the promotion of protein synthesis via p70 ribosomal S6 kinase (S6K) and initiation factor 4E-binding protein 1 (4E-BP1) phosphorylation (White and Kahn 1994; Dupont et al. 2009). Additionally, PKB can be internalized into the nucleus of the cells acting as a transcription factor, which process – together with the MAP-kinase pathway – also play pivotal role in the growth promoting effect of insulin and Insulin-like Growth Factor-1 and -2 (White and Kahn 1994). PKB proved to play an inhibitory role in the regulation of MyoD and myogenin mRNA expression, resulting in the stimulation of myoblast proliferation but not

differentiation *in vitro* (Sato et al. 2012). This observation was reinforced *in vivo* by improved growth performance, after insulin administration to newly hatched chicks (Sato et al. 2012).

Improved insulin sensitivity of tissues, and especially of skeletal muscle leads to better production parameters, ameliorated muscle mass development, as well as to more favorable feed utilization in birds, corroborated by Józefiak et al. (2010), who found that multi-carbohydrase and phytase enzyme supplemented feed promoted the hepatic IR expression and optimized feed utilization of broiler chicken. Stimulated microbial SCFA, and especially butyrate production was assumed to be the reason for reduced body fat mass, elevated GLP-1 production and ameliorated insulin sensitivity in mice fed NSP-rich, barley-based diet (Miyamoto et al. 2018). Wheat-based diet (rich in soluble NSPs) was also reported to decrease plasma insulin concentration and to increase the protein abundance of certain insulin signaling proteins in the liver and adipose tissue of broilers (Kulcsár et al. 2016).

In the skeletal muscles, an age-related decrease in the sensitivity of insulin signaling proteins to nutritional factors was described both in rats (Gupte et al. 2008) and birds (Deng et al. 2014). The declination of insulin sensitivity in the skeletal muscles of chicken might be explained with the downregulation of mTOR, S6K and 4E-BP1 expression and phosphorylation with aging, manifested by attenuated muscle growth capacity (Deng et al. 2014).

2.6.4. Glucagon homeostasis and the glucagon signaling pathway

Beside insulin, carbohydrate metabolism of birds is primarily navigated by its potent antagonist glucagon, secreted by the α-islet cells in a much higher quantity, compared to insulin production (Ruffier et al. 1998). This 29 amino acid long hormone stimulates the process of gluconeogenesis and glycogenolysis in the liver, thus is responsible for the maintenance of the unusually high plasma glucose level of birds (Hazelwood 1973; Roach 1990).

On the surface of the target cells, glucagon binds to its G-protein coupled glucagon receptor (GCGR; Jelinek et al. 1993), which is bound to G-protein $_s\alpha$ and q subunits ($G_s\alpha$ and G_q). $G_s\alpha$ activates adenylate cyclase enzyme, catalyzing the formation of cAMP, which activates protein kinase A (PKA). Same time, phospholipase C (PLC) is also activated by G_q, increasing the concentration of inositol 1,4,5-trisphosphate and intracellular calcium level (Christophe 1995; Burcelin et al. 1996). The role of PKA is partly to activate glycogen phosphorylase through the phosphorylation of glycogen phosphorylase kinase. Glycogen phosphorylase catalyzes the formation of glucose-6-phosphate from glycogen, which is the first step of glycogenolysis, before glucose-6-phosphate is dephosphorylated to glucose by glucose-6-phosphatase to increase blood sugar level (Krebs 1980). This enzyme can also be activated by glucagon (Band and Jones 1980). On the other hand, PKA also activates glycogen synthase, which means inactivation in case of this latter enzyme (Ramachandran et al. 1983; Ciudad et al. 1984), responsible for hepatic glycogenesis.

Apart from acting on glycogen homeostasis, PKA stimulates gluconeogenesis over glycolysis by supporting the action of fructose 1,6-bisphosphatase via the activation of fructose-2,6-bisphosphatase (Kurland and Pilkis 1995; Pilkis et al. 1982), and by stimulating the production and activity of glucose-6-phosphatase enzyme (Okar and Lange 1999; Pilkis et al. 1982; Striffler et al. 1984).

The main target of glucagon is the liver, where avian GCGR is highly expressed, but practically all tissues of chicken show certain expression, including whole brain, heart, pancreas, spleen, kidneys, lung, muscles, adipose tissue, reproductive organs and the small intestines (Wang et al. 2008). Despite insulin sensitivity, the responsiveness of the tissues to glucagon hormone is increasing with aging in chicken (Joseph et al. 1996).

2.7. The avian protein metabolism

The avian protein metabolism in general is similar to that of mammalian species; however, some important differences exist.

The protein supply of the birds is mostly covered from dietary proteins, but their utilization depends on the amount, composition and digestibility. Protein digestion starts in the proventriculum, after partial denaturation in the crop by acidogenic bacteria (Vilela et al. 2020). The action of pepsin and hydrochloric acid in the proventriculum is followed by the mechanistic mixing and grinding in the gizzard. Then protein digestion is completed by pancreatic (chymotrypsin, trypsin, elastase, collagenase, carboxypeptidases) and intestinal proteolytic enzymes (aminopeptidases) and ends by the absorption of the produced amino acids and peptides in the small intestines by a series of amino acid transporters and PepT1 di- and tripeptide transporter (Adibi et al. 1967; Gal-Garber and Uni 2000; Gilbert et al. 2007). The expression rate of the amino acid transporters of chickens increases progressing down the small intestines and with aging (Gilbert et al. 2007). Amino acids, and to a lesser extent, some peptides – that are not consumed intracellularly – are transported through the basolateral membrane of the enterocytes by the aid of simple or facilitated diffusion, or active transport systems (Matthews 2000; Hou et al. 2017; Qaid and Al-Garadi 2021), then delivered to the liver by the portal circulation. Here most is used for hepatic protein synthesis and is forwarded into the systemic circulation as free proteins available for extrahepatic tissues.

Amino acids are the substantial precursors of the synthesis of – amongst others – any kind of proteins, but they also can be catabolized for energy production, or to gain glucose in the gluconeogenesis. In birds, the most important sites of protein synthesis are the liver, the muscles and the reproductive organs, with uncommonly high protein requirement for muscle, egg and feather production (Sturkie's Avian Physiology 2014). The first four limiting amino acids for birds are lysine, methionine, threonine and tryptophan. The whole-body protein synthesis, particularly muscle protein synthesis is markedly depressed under starvation or by inadequate

amino acid supply, especially in case of limiting amino acid scarcity (Uzu 1983; Kino and Okumura 1987). *In vitro* studies indicate that both insulin and insulin-like growth factor stimulate chicken amino acid uptake and protein synthesis, whereas inhibit protein degradation in muscles (Duclos et al. 1993), and also *in vivo* in human (Bonadonna et al. 1993). In this aspect, glucagon can be considered as a hormone synergistic with insulin in promoting plasma amino acid clearance and postprandrial amino acid disposal by stimulating hepatic amino acid uptake, catabolism and ureagenesis (Holst et al. 2017).

Avian proteins do not have an amino acid reserve function, therefore, all surplus amino acids supplied in the diet are catabolized. Unlike in mammals, the major end-product of the degradation of nitrogen containing compounds (amino acids and purines) is uric acid, but to a lesser extent, ammonia and urea are also urinary nitrogenous wastes in birds (Stevens 1996). The predominant organ responsible for uric acid formation in the uric acid cycle is the liver, but kidneys also contribute to the formation of this deleterious end-product by c.a. 17% in chicken (Chin and Quebbemann 1978; McFarland and Coon 1984). Despite that uricase enzyme, responsible for the conversion of uric acid to allantoin lacks in birds (de Boeck and Stockx 1978), the plasma of chicken and turkey contains considerable amount of allantoin, highlighting the role of uric acid as antioxidant in these species (Simoyi et al. 2003).

The traditional poultry feed formulation has been based on the crude protein concept, sometimes providing an amino acid composition not meeting the real needs of the birds. Apart from the economic consequences of this practice, undigested proteins and the derivatives of absorbed, but excess amino acids become the precursor of uric acid formation, which undergoes bacterial decomposition to ammonia after excretion, affecting animal welfare and being environmental question same time. Intensive animal production is considered as main contributor to nitrogen pollution of the environment within the agricultural system, therefore, reduction of the nitrogen excretion of poultry via dietary manipulations, such as feeding low-protein, essential amino-acid fortified diets is desirable (Donsbough et al. 2010). Apart from environmental approach, another disadvantage of protein overfeeding is that the excretion of the degradation products of excess amino acids requires energy expenditure, and thus compromises the performance of the animals (Buteri 2003; Barzegar et al. 2020).

With the ability of industrial amino acid production, the theoretical possibility of ideal protein and amino acid supply of animals raised, that achieves optimal balance of amino acids, fulfilling the requirements of the birds for maintenance and satisfactory protein utilization, minimizing the use of amino acids as energy source and nitrogen excretion (Donsbough et al. 2010). Literature data indicate that the application of low-protein diets with concomitant essential amino acid supplementation does not impair the performance of chickens (Darsi et al. 2012).

3. Significance and aims of the study

Constantly growing human population is seeking for the opportunities of satisfactory poultry meat production from ecological, economical, nutritional, human health and animal welfare point of view as well. Therefore, there are efforts in finding new ways of growth promotion, such as alternative feed additive SCFAs, especially butyrate, and new concepts in diet formulation have also appeared, aiming optimal composition for better growth and reducing costs while decreasing nitrogen load of the environment. However, there is still little knowledge on how the recently applied dietary strategies might affect the general metabolic health of the broiler chicken, or influence meat composition, and unfortunately scientific results are sometimes rather contradictory.

The aim of our research group was to examine the effects of butyrate – widely used in both pig and poultry farming as a potent alternative to antibiotics in growth promoting – of exogen and endogen origin, in combination with distinct dietary crude protein levels, also assessing possible age-dependency of broiler chickens.

In **Study I**, the investigation of the effects of distinct dietary crude protein levels (normal vs. reduced, the latter supplemented with limiting amino acids), combined or not with sodium (n)-butyrate as feed additive, together with different grains as bases of diets (wheat vs. maize, highly distinct in their soluble NSP content and therefore, in the ability to enhance cecal butyrate production) was aimed on broilers at the age of 7, 21, and 42 days (d). With the measurement of the peripheral blood concentration or activity of ten selected markers of carbohydrate, nitrogen, lipid metabolism and hormonal homeostasis, our purpose was to gain a complex overview on how these above mentioned nutritional factors might influence the general metabolic state of chickens in relation with their age, contributing to the improvement of scarce literature data on the avian physiology under special feeding conditions as well.

Based on the literature data and the results of Study I – where avian metabolism proved to be the most responsive to the investigated dietary factors in the phase of intensive growth – , d 21 was considered optimal for the assessment of the responsiveness of certain glucagon and insulin signaling key elements in the liver to dietary factors, on both gene expression and protein abundance level (**Study II**). The importance of the investigation is highlighted by the fact that deeper understanding of the avian glucose homeostasis and the ways of its modulation might lead to the amelioration of insulin sensitivity, thus the improvement of productivity and animal welfare in broilers.

Regarding that the quality and amount of meat is outstandingly important for the broiler industry, we tested whether the changes measured in Study I and II led to detectable and appreciable results in meat quantity or quality by the age of slaughter (d 42, **Study III**). Taking into account that protected and unprotected forms of butyrate are equally applied in poultry

nutrition, this study was designed with the inclusion of several types of protected butyrate and unprotected sodium (n)-butyrate with the commonly used maize-based diet formulation with lowered or normal dietary crude protein level, trying to approach general practice and to modelling real farm conditions the best.

Beside the production of appropriate amount and quality of meat, safety of chicken endproducts is also an indispensable requirement and aim of the broiler sector, therefore, an *in vitro* experiment was designed and performed to test the antimicrobial efficacy of sodium (n)-butyrate on various *Campylobacter jejuni* isolates at two pH values by colony counting, together with the ampicillin and enrofloxacin sensitivity of the strains (**Study IV**). As previous studies of the research group proved that in case of applying wheat-based diet, cecal butyrate content could reach notable concentration by the age of 42 days, we aimed to investigate the possible beneficial effects of butyrate in the reduction of *Campylobacter* colonization by slaughtering, from theoretical aspects, hopefully contributing to the extension of the application of butyrate and gaining valuable background information for *Campylobacter* eradication programs.

Summarizing the above detailed goals, the main aims of this PhD study were:

Ad1, to monitor a set of biochemical blood plasma parameters, reflecting the age-related responsiveness of the main processes of the avian intermediary metabolism, evoked by the type of dietary cereal (wheat vs. maize), crude protein content (normal vs. reduced by 15% and fortified with limiting amino acids) and sodium (n-)butyrate supplementation (1.5 g/kg diet vs. no supplementation) in broiler chickens.

Ad2, to investigate how the gene and protein expression of selected prominent members of hepatic insulin and glucagon signaling are influenced by the above detailed nutritional factors in the phase of intensive growth.

Ad3, to gain information on the possible changes of carcass traits and the chemical composition of meat induced by butyrate of different types (free sodium (n-)butyrate salt vs. various protected forms), as well as dietary crude protein level (normal vs. reduced by 15% and fortified with limiting amino acids) with maize-based diets.

Ad4, to test the *in vitro* antibacterial effect of sodium (n)-butyrate against *Campylobacter jejuni* strains.

4. Materials and methods

4.1. Ethic statement

The results of **Study I, II** and **III** originate from one large-scale experiment, where animal welfare considerations led to the concept of collecting the most possible data from the least number of animals.

Experimental procedures, including housing, alimentation, treatment and slaughter were approved by the Government Office of Pest County, Food Chain Safety, Plant Protection and Soil Conservation Directorate, Budapest, Hungary (number of permission: PEI/001/1430-4/2015) and was conducted in strict accordance with the directive no. 2010/63/EU on the protection of animals used for scientific purposes, as well as with the government decree no. 40/2013 (II. 14.) on animal experiments.

4.2. *In vivo* studies

4.2.1. Animals and treatments

Three hundred and ten newly hatched male Ross 308 broiler chicks (*Gallus gallus domesticus*) were purchased from a commercial hatchery (Gallus Company, Devecser, Hungary) and were randomly classified to eight dietary groups (n = 10 per sampling point per group, n = 30 in total per group) for **Study I** and **II**. Further, seven dietary groups were formed for **Study III** (n = 10 per group). Each group showed similar average body weights on day 1.

Environmental conditions met the Ross recommendations (Aviagen 2014) and rearing technology was set as follows. Upon arrival, the animals were housed on wheat straw bedding in metal framed floor pens in the Institute of Physiology and Nutrition, Hungarian University of Agriculture and Life Sciences (Herceghalom, Hungary). The whole house temperature was set to 30 ºC, then lowered to 28 ºC by decreasing it with 1 ºC/day, later by 1 ºC/3 days steps until 22 ºC was reached on d 21 and maintained afterwards. The relative air humidity was set between 60-70% on the first 3 days of housing, then kept above 50% throughout the experimental period (controlled daily with hygrometer). The light intensity was set to 30-40 lux in the broiler house with 23 h light and 1 h dark period during the first week of life, then lowered to 10 lux with 20 h light and 4 h continuous dark period. Feed and drinking water were available *ad libitum* thorough the entire study. Animals were monitored daily, and showed no signs of discomfort or illness in any of the dietary groups. Uniform management and vaccination schedule were followed for all the birds, feed intake and growth performance matched the parameters detailed in the Broiler Management Handbook: Ross 308 (Aviagen 2014).

Dietary treatments followed a 2 x 2 x 2 factorial arrangement, forming eight dietary groups as follows for **Study I** and **II**. Two different basal diets were applied (maize-based [**MB**] or wheat-based [**WB**] diet), with or without sodium butyrate supplementation in the commonly used dose in poultry nutrition (1.5 g/kg diet; **But** vs. **Ctr** groups), already successfully applied in our earlier studies (Kulcsár et al. 2016 and 2017). Further, the diet of four groups was formulated with crude protein content matching standard recommendations of the appropriate dietary phase ("normal protein" [**NP**] groups with 22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets), while four groups was fed a diet with crude protein content reduced by 15% ("low protein" [**LP**] groups with 19.1%, 18.0% and 16.0% crude protein, respectively). Amino acid levels in all diets were calculated, and the four first-limiting commercially available amino acids (L-lysine hydrochloride, DL-methionine, L-threonine and L-tryptophan) were supplemented to "LP" diets to meet the recommendations of the breeder (Aviagen 2014).

Regarding **Study III**, animals of seven dietary groups were used for sampling, where the dietary regime applied for four groups was identical with that of the four MB groups of Study I and II, further, three additional groups were formed for Study III only. Summarizing the design, maize was used as bases of diets for all the seven dietary groups (**MB** diets). Five groups of chickens were fed diets with normal dietary crude protein level of the appropriate dietary phase, while two groups received low-protein, limiting amino-acid supplemented diet ([**NP**] and [**LP**] groups, respectively). Crude protein levels of diets were set and limiting amino acid addition was performed for LP diets as described above at Study I and II. Further, the feed of two groups was completed with unprotected sodium (n)-butyrate (1.5 g/kg diet; **But**), and different forms of protected sodium butyrate were blended into the diet of three NP groups as follows: a highly concentrated, film-coated sodium butyrate (Intest-Plus S90 with 90% sodium butyrate content, in the dose of 1.0 g/kg diet [pure sodium butyrate content: 0.9 g/kg diet)]; **NP S90** group), and vegetable fat-embedded sodium butyrate products with various butyrate contents (Intest-Plus SC40 with 40% sodium butyrate content, in the dose of 1.5 g/kg diet [pure sodium butyrate content: 0.6 g/kg diet]; **NP SC40** group, as well as IntestPlus SC30 with 30% sodium butyrate content, in the dose of 2.0 g/kg diet [pure sodium butyrate content: 0.6 g/kg diet]; **NP SC30** group). Doses were set according to the manufacturer's instructions. Groups without any form of butyrate supplementation were regarded as controls (**Ctr**).

An overview of the experimental groups is presented in **Table 3**.

Table 3. Summary of dietary groups of Study I-III.

Study	Abbreviation of group	Cereal type (basal diet)	Crude protein content	Sodium butyrate supplementation (g/kg diet)	Protected butyrate supplementation (g/kg diet)
I, II, III	MB NP Ctr	Maize	adequate („normal")	no	no
I, II, III	MB NP But	Maize	adequate („normal")	1.5	no
I, II, III	MB LP Ctr	Maize	reduced („low")	no	no
I, II, III	MB LP But	Maize	reduced („low")	1.5	no
I, II	WB NP Ctr	Wheat	adequate („normal")	no	no
I, II	WB NP But	Wheat	adequate („normal")	1.5	no
I, II	WB LP Ctr	Wheat	reduced („low")	no	no
I, II	WB LP But	Wheat	reduced („low")	1.5	no
III	MB NP S90	Maize	adequate („normal")	no	1.0
III	MB NP SC40	Maize	adequate („normal")	no	1.5
III	MB NP SC30	Maize	adequate („normal")	no	2.0

MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation; S90: Protected butyrate supplementation of the diet (Intest-Plus S90, 1.0 g/kg diet); SC40: Protected butyrate supplementation of the diet (Intest-Plus SC40, 1.5 g/kg diet); SC30: Protected butyrate supplementation of the diet (Intest-Plus SC30, 2.0 g/kg diet).

For **Study I**, **II** and **III**, starter diets were switched to growers on d 10 and growers to finisher diets on d 25. All diets were set isoenergetic and isonitrogenous within a phase, designed to suit nutrient specifications of Ross 308 recommendations (Aviagen 2014) and fed in mash form. The diets were formulated and produced by the feed mixing facility of the Hungarian University of Agriculture and Life Sciences. Unprotected sodium (n)-butyrate was purchased from Sigma-Aldrich (Darmstadt, Germany), while protected butyrate products were obtained from Palital Feed Additives (Velddriel, the Netherlands). The soluble arabinoxylan content was previously determined as 0.88 mg/g for maize and 9.37 mg/g for wheat (measurements performed in the Agricultural Institute, Center for Agricultural Research, Martonvásár, Hungary following the method of Douglas [1981]. Compositions and calculated nutrient contents of diets of all dietary phases (without sodium [n-]butyrate supplementation) are indicated in **Table 4-6**.

Table 4. Ingredients and calculated nutrient composition of experimental broiler starter diets, without sodium butyrate supplementation

Ingredients	Unit	Maize-based Normal CP	Maize-based Low CP	Wheat-based Normal CP	Wheat-based Low CP
Maize	%	57.60	61.00	0.00	0.00
Wheat	%	0.00	0.00	54.79	62.60
Solvent extr. soybean meal	%	27.00	28.00	31.00	26.48
PL-68†	%	6.50	0.00	3.00	0.00
Sunflower oil	%	3.50	3.50	6.00	5.30
Wheat bran	%	0.00	1.72	0.00	0.00
Limestone	%	1.70	1.60	1.70	1.70
MCP	%	1.80	2.00	1.70	1.70
Salt (NaCl)	%	0.40	0.40	0.40	0.40
L-lysine hydrochloride	%	0.44	0.58	0.38	0.60
DL-methionine	%	0.43	0.44	0.41	0.45
L-threonine	%	0.09	0.22	0.11	0.26
L-tryptophan	%	0.04	0.04	0.00	0.00
Vitamin and mineral premix‡	%	0.50	0.50	0.50	0.50
Axtra XB 201 enzyme§	%	0.00	0.00	0.015	0.015
Calculated values					
Dry matter	%	89.65	89.32	89.78	89.47
Crude protein	%	22.02	18.65	22.05	18.76
Soluble NSP	mg/kg	506.88	536.80	5133.82	5865.62
ME	MJ/kg	12.65	12.61	12.63	12.62
Ether extract	%	6.54	6.30	7.49	6.62
Crude fiber	%	2.51	2.74	2.88	2.81
Ash	%	6.97	7.23	7.37	7.42
Lysine	%	1.43	1.43	1.44	1.43
Methionine + Cysteine	%	1.07	1.05	1.08	1.07
Threonine	%	0.97	0.94	0.94	0.94
Tryptophan	%	0.23	0.25	0.26	0.24
Arginine	%	1.17	1.24	1.34	1.22
Isoleucine	%	0.74	0.78	0.85	0.78
Leucine	%	1.59	1.68	1.52	1.41
Valine	%	0.83	0.88	0.93	0.86
Total Ca	%	1.15	1.15	1.16	1.14
Total P	%	0.79	0.80	0.82	0.80
Available P	%	0.54	0.53	0.56	0.54

CP: Crude protein; MCP: Monocalcium phosphate; ME: Metabolizable energy; NSP: Non-starch polysaccharide.

†Protein concentrate, by-product of glutamic acid production from bacterial biomass (KJK-Agroteam Ltd., Dombóvár, Hungary).

‡Provides per kilogram of diet: vitamin A 12013 IU; vitamin D3 3875 IU; vitamin K 3.3 mg; vitamin E 46.5 IU; vitamin B1 2.33 mg; vitamin B2 7.44 mg; vitamin B6 3.88 mg, vitamin B12 0.016 mg; calcium pantothenate 13.95 mg; folic acid 1.56 mg; niacin 46.5 mg; choline chloride 504 mg; Fe 60 mg; Mn 100 mg; Cu 12.5 mg; Zn 83 mg; Se 0.42 mg; Co 0.28 mg; I 1.25 mg.

§Enzymatic activity in the product (DuPont Animal Nutrition, New Century, KS, USA) 12200 U/g endo-1,4-β-xylanase and 1520 U/g endo-1,3(4)-β-glucanase.

Table 5. Ingredients and calculated nutrient composition of experimental broiler grower diets, without sodium butyrate supplementation

Ingredients		Maize-based Normal CP	Maize-based Low CP	Wheat-based Normal CP	Wheat-based Low CP
Maize	%	60.71	65.31	0.00	0.00
Wheat	%	0.00	0.00	61.30	66.56
Solvent extr. soybean meal	%	22.20	24.54	19.31	20.01
PL-68†	%	8.00	1.00	8.50	2.50
Sunflower oil	%	4.80	4.50	6.70	6.50
Wheat bran	%	0.00	0.00	0.00	0.00
Limestone	%	1.30	1.20	1.35	1.35
MCP	%	1.35	1.60	1.15	1.15
Salt (NaCl)	%	0.40	0.40	0.40	0.40
L-lysine hydrochloride	%	0.34	0.41	0.38	0.48
DL-methionine	%	0.36	0.37	0.35	0.38
L-threonine	%	0.00	0.15	0.05	0.16
L-tryptophan	%	0.04	0.02	0.00	0.00
Vitamin and mineral premix‡	%	0.50	0.50	0.50	0.50
Axtra XB 201 enzyme§	%	0.00	0.00	0.015	0.015
Calculated values					
Dry matter	%	89.72	89.34	89.90	89.55
Crude protein	%	21.12	17.85	21.10	17.89
Soluble NSP	mg/kg	534.25	574.73	5743.81	6236.67
ME	MJ/kg	13.27	13.24	13.24	13.24
Ether extract	%	7.96	7.39	8.45	7.92
Crude fiber	%	2.34	2.48	2.51	2.61
Ash	%	5.78	6.03	6.00	6.13
Lysine	%	1.25	1.22	1.25	1.22
Methionine + Cysteine	%	0.96	0.95	0.94	0.95
Threonine	%	0.84	0.84	0.85	0.81
Tryptophan	%	0.21	0.20	0.20	0.21
Arginine	%	1.01	1.11	0.97	1.02
Isoleucine	%	0.65	0.72	0.62	0.65
Leucine	%	1.45	1.58	1.14	1.20
Valine	%	0.74	0.81	0.70	0.74
Total Ca	%	0.92	0.93	0.90	0.90
Total P	%	0.68	0.69	0.71	0.67
Available P	%	0.45	0.45	0.49	0.44

CP: Crude protein; MCP: Monocalcium phosphate; ME: Metabolizable energy; NSP: Non-starch polysaccharide.

†Protein concentrate, by-product of glutamic acid production from bacterial biomass (KJK-Agroteam Ltd., Dombóvár, Hungary).

‡Provides per kilogram of diet: vitamin A 12013 IU; vitamin D3 3875 IU; vitamin K 3.3 mg; vitamin E 46.5 IU; vitamin B1 2.33 mg; vitamin B2 7.44 mg; vitamin B6 3.88 mg, vitamin B12 0.016 mg; calcium pantothenate 13.95 mg; folic acid 1.56 mg; niacin 46.5 mg; choline chloride 504 mg; Fe 60 mg; Mn 100 mg; Cu 12.5 mg; Zn 83 mg; Se 0.42 mg; Co 0.28 mg; I 1.25 mg.

§1Enzymatic activity in the product (DuPont Animal Nutrition, New Century, KS, USA) 12200 U/g endo-1,4-β-xylanase and 1520 U/g endo-1,3(4)-β-glucanase.

Table 6. Ingredients and calculated nutrient composition of experimental broiler finisher diets, without sodium butyrate supplementation

Ingredients		Maize-based Normal CP	Maize-based Low CP	Wheat-based Normal CP	Wheat-based Low CP
Maize	%	63.66	70.25	0.00	0.00
Wheat	%	0.00	0.00	64.69	69.69
Solvent extr. soybean meal	%	24.50	20.29	19.35	19.35
PL-68†	%	3.00	0.70	5.00	0.00
Sunflower oil	%	5.00	4.30	6.96	6.90
Wheat bran	%	0.00	0.00	0.00	0.00
Limestone	%	1.09	1.09	1.35	1.26
MCP	%	1.40	1.60	1.15	1.15
Salt (NaCl)	%	0.40	0.40	0.40	0.40
L-lysine hydrochloride	%	0.19	0.39	0.25	0.32
DL-methionine	%	0.26	0.33	0.30	0.31
L-threonine	%	0.00	0.13	0.08	0.15
L-tryptophan	%	0.00	0.02	0.00	0.00
Vitamin and mineral premix‡	%	0.50	0.50	0.50	0.50
Axtra XB 201 enzyme§	%	0.00	0.00	0.015	0.015
Calculated values					
Dry matter	%	89.46	89.21	89.70	89.40
Crude protein	%	19.04	16.13	19.07	16.20
Soluble NSP	mg/kg	560.21	618.2	6061.45	6529.95
ME	MJ/kg	13.41	13.41	13.38	13.44
Ether extract	%	7.96	7.27	8.51	8.17
Crude fiber	%	2.47	2.36	2.56	2.62
Ash	%	5.46	5.65	5.83	5.80
Lysine	%	1.09	1.08	1.07	1.02
Methionine + Cysteine	%	0.86	0.87	0.87	0.86
Threonine	%	0.74	0.74	0.79	0.72
Tryptophan	%	0.18	0.18	0.20	0.21
Arginine	%	1.11	0.99	0.99	1.01
Isoleucine	%	0.71	0.64	0.63	0.65
Leucine	%	1.56	1.48	1.16	1.19
Valine	%	0.80	0.74	0.71	0.74
Total Ca	%	0.85	0.87	0.90	0.87
Total P	%	0.66	0.68	0.69	0.66
Available P	%	0.42	0.44	0.46	0.42

CP: Crude protein; MCP: Monocalcium phosphate; ME: Metabolizable energy; NSP: Non-starch polysaccharide.

†Protein concentrate, by-product of glutamic acid production from bacterial biomass (KJK-Agroteam Ltd., Dombóvár, Hungary).

‡Provides per kilogram of diet: vitamin A 12013 IU; vitamin D3 3875 IU; vitamin K 3.3 mg; vitamin E 46.5 IU; vitamin B1 2.33 mg; vitamin B2 7.44 mg; vitamin B6 3.88 mg, vitamin B12 0.016 mg; calcium pantothenate 13.95 mg; folic acid 1.56 mg; niacin 46.5 mg; choline chloride 504 mg; Fe 60 mg; Mn 100 mg; Cu 12.5 mg; Zn 83 mg; Se 0.42 mg; Co 0.28 mg; I 1.25 mg.

§1Enzymatic activity in the product (DuPont Animal Nutrition, New Century, KS, USA) 12200 U/g endo-1,4-β-xylanase and 1520 U/g endo-1,3(4)-β-glucanase.

4.2.2. Samplings

Although neither of the studies was designed to assess the performance of the broilers, weights of animals were recorded at day-old (upon arrival) and at the age of 7, 21 and 42 days to gather background data and samples were collected as follows.

For **Study I**, peripheral blood samples were gained on d 7, 21 and 42 by puncture of the brachial vein of ten randomly selected chickens per experimental group at every time point, in order to follow the possible age-dependent effects of the investigated nutritional factors. Selection of animals and samplings were strictly performed between 4.00 – 7.00 pm to minimize diurnal variation, by taking one chicken randomly from each group and then repeating the procedure until ten samples per group were obtained. Blood was collected in heparinized tubes, kept on ice until the immediate on the spot separation of blood plasma by centrifugation (2000 g, 10 min, 4 ºC), then shock frozen in liquid nitrogen and stored at -80 ºC until further processing.

For **Study II**, after body weight measurement and blood sampling, chickens were decapitated in CO_2 narcosis on d 21, the liver was exsanguinated with chilled sterile physiological saline solution by the cannulation of the *v. pancreaticoduodenalis*, then excised and tissue samples were gained for q-PCR and Western blot analyses. PCR samples were taken and placed into RNA isolation reagent (easy-BLUE™, Sigma-Aldrich, Darmstadt, Germany) and placed on dry ice, while Western blot samples were shock frozen in liquid nitrogen, then all the samples were stored at -80 ºC until processing.

For **Study III**, chickens were slaughtered in CO_2 narcosis on d 42 by decapitation, then carcass weight (including skin and wings, excluding giblets), deboned breast meat yield, femoral muscle weight, and the weights of liver, heart, spleen and abdominal adipose tissue were measured. Additionally, representative samples (60 g tissue from the same anatomic site) were taken from the pectoral (*m. pectoralis major*) and femoral (*m. iliotibialis*) muscle for chemical analysis of meat composition. Muscle samples were minced, freeze-dried in liquid nitrogen, ground and stored at -20 ºC until further processing.

4.2.3. Measurements

4.2.3.1. Study I – Plasma measurements

In **Study I**, plasma concentrations of total protein, albumin, uric acid, glucose, triglyceride, GLP-1, GIP, insulin, and activity of aspartate aminotransferase and Creatine Kinase enzymes were measured to investigate metabolic and hormonal changes induced by nutritional factors.

After thawing the samples on ice, plasma concentrations of total protein, albumin and uric acid, as well as aspartate aminotransferase and creatine kinase activities were estimated by spectrophotometric measurements with an automated apparatus (Olympus AU400 Chemical

Analyzer, Beckman Coulter, Brea, CA, USA). Reagents were purchased from Diagnosticum (Budapest, Hungary), or in case of uric acid, from Dialab (Budapest, Hungary).

GLP-1, GIP and insulin levels were measured by chicken-specific sandwich ELISA tests (MyBioSource, San Diego, CA, USA; catalogue number: MBS260694 [GLP-1], MBS261884 [GIP], MBS2516351 [insulin]), as instructed by the manufacturer, with incubations at 37 ºC in all cases. Briefly, after thawing on ice, 90 µl plasma sample or serially diluted standard solution was measured into each well of the microtiter plate, incubated for 90 min, then fluid was removed and wells were flushed three times with Wash Solution (or in case of insulin, no washing was prescribed). As a next step, the plate was incubated for 60 min with 100 µl biotinylated chicken (GLP-1, GIP or insulin specific) antibody solution, then fluid was discarded and wells were washed three times again. Further, we measured 100 µl avidin-conjugated horseradish peroxidase enzyme liquid in the wells, incubated the plates for 30 min, removed the fluid and washed the wells five times. Finally, 100 µl (or 90 µl for insulin determination) color reagent liquid was added to the wells and incubated until properly distinguishable shades of blue color appeared. At this point, the reaction was stopped with 100 µl (or in case of insulin, 50 µl) sulphuric acid containing stop solution, and the optical density of the yellow color – proportional to the concentration of the investigated parameter – was immediately measured at 450 spectrophotometrically.

Glucose and triglyceride concentrations were determined by colorimetric methods using Glucose GOD/PAP and Triglyceride PAP liquid reagents (Diagnosticum, Budapest, Hungary; catalogue number: 46862 [glucose], 47162 [triglyceride]), following the instructions of the producer. Briefly, first, 3µl sample or serially diluted glucose/triglyceride standard solution was measured onto chilled microtiter plates in triplicate, followed by 300 µl color reagent. After incubation for 5 min (37 ºC), the intensity of the red quinoneimine endproduct positively correlated with the concentration of the investigated parameter in the sample, measured immediately at 505 nm with ice-cold plates.

4.2.3.2. Study II – Messenger RNA isolation, reverse transcription and q-PCR measurements

In **Study II**, gene expression of GCGR, IRβ and mTOR was assessed by measuring the mRNA concentration of the samples by q-PCR method after reverse transcription, whereas protein abundance of the same members of carbohydrate homeostasis was evaluated by Western blotting.

Reagents were obtained from Reanal (Budapest, Hungary) for reverse transcription and q-PCR measurements, and purchased from Sigma-Aldrich (Munich, Germany) for Western blotting, except when otherwise specified.

Messenger RNA isolation and reverse transcription

During the process of mRNA isolation and reverse transcription, all centrifugations were performed at 4 ºC. After thawing on ice, tissue samples were aseptically homogenized by a tissue grinder Potter-Elvehjem homogenizer, centrifuged (Beckman-Coulter, Indianapolis, IN, USA; 12000 g, 10 min), then the middle homogenous phase was centrifuged again (12000 g, 10 min). After incubation of the clean supernatant for 5 min at room temperature, 200 µL chloroform was added into the tubes followed by vigorous shaking, then another incubation for 5 min at room temperature was applied and samples were centrifuged (13000 g, 10 min) for phase separation. Thereafter, aqueous top phase was mixed with equal amount of isopropanol, shaken, incubated for 10 min at room temperature and centrifuged (13000 g, 5 min). Afterwards, 1 mL 75% ethanol was measured onto the RNA pellet, followed by subsequent centrifugation (10000 g, 5 min), then the pellet was dried for 10 min at 60 ºC, dissolved in 50 µL molecular biology grade water, incubated for 10 min at 60 ºC and finally the mRNA concentration of the sample was determined by spectrophotometry (NanoDrop Spectrophotometer, ThermoFisher Scientific, Waltham, MA, USA; software version: ND-1000 V3.5.1). Removal of genomic DNA was performed with RapidOut DNA Removal Kit (Thermo Scientific, Waltham, MA, USA) using a volume of sample containing 1000 ng mRNA, synthesis of cyclic deoxyribonucleic acid (cDNA) was implemented with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with random hexamer primer, according to the instructions of the manufacturer.

q-PCR measurements

The q-PCR measurements were performed with a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany; software version 2.1.0, Build 9), by the aid of Thermo Scientific Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific), containing Taq DNA polymerase, uracil-DNA glycosylase, deoxynucleotide triphosphates (with deoxyuridine triphosphate), SYBR Green I in an optimized PCR buffer with blue dye. For the reaction, 10 µl Master Mix, 0.5 µl sample (set to 2.5 ng/µl cDNA concentration) 1 µl forward and reverse primer, and molecular biology grade water (up to 20 µl total volume) were mixed, with no sample in case of blank. Primer pairs were designed with NCBI Primer-BLAST and purchased from Biocenter, applied as detailed in **Table 7** to test genes of interest. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as reference gene; its expression was unaffected by any of the applied dietary treatments. The temperature profile of the reaction was set as follows: uracil-DNA glycosylase treatment at 50 ºC for 2 min; initial denaturation at 95 ºC for 10 min; denaturation step at 95 ºC for 15 s; annealing at 60 ºC for 30 s and extension at 72 ºC for 30 s for 40 cycles.

At the end of each cycle, fluorescence monitoring was set for 10 s. Relative gene expressions were calculated by the $2^{-\Delta\Delta Ct}$ method applying the software 2.1.0 (Build 9).

Table 7. Primer pairs used to test genes of interest

Gene	Primer	Primer sequence	Primer efficacy	Amplicon size	NCBI accession	Threshold
GAPDH	Forward (5'-3')	GGGCACGCCATCACTATCTT	0.9408	187	NM 204305.1	0.03
	Reverse (5'-3')	TCACAAACATGGGGGCATCA				
GCGR	Forward (5'-3')	ATCCCGTGGGTTGTTGTGAA	0,932	195	NM 001101035.1	0.02
	Reverse (5'-3')	CTTGTAGTCGGTGTAGCGCA				
IRβ	Forward (5'-3')	CAACCCACACTGGTGGTCAT	0.9376	134	XM 001233398.5	0.0036
	Reverse (5'-3')	GCAGCCATCTGGATCATTTCTC				
mTOR	Forward (5'-3')	GTGGCGATCCTATGGCATGA	0.9784	276	XM 417614.6	0.05
	Reverse (5'-3')	ACGCCTGAAAACGTGGTAGT				

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase (reference gene); GCGR: Glucagon receptor; IRβ: Insulin receptor β subunit; mTOR: Mammalian target of rapamycin. Threshold was calculated by software.

4.2.3.3. Study II – Western blot measurements

The protein abundance of GCGR, IRβ and mTOR was determined from liver samples by semiquantitative Western blotting in duplicates. Western blot measurements were implemented at the University of Hohenheim, Institute of Animal Science (Hohenheim, Germany).

Approximately 300 mg liver sample was completed with 0.6 mL lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [Carl Roth GmbH, Karlsruhe, Germany], 4 mM ethylene glycol-bis[2-aminoethylether]-N,N,N,N'-tetraacetic acid, 10 mM ethylenediaminetetraacetic acid, 0.1% Triton X-100, 100 mM β-glycerol phosphate, 15 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 2.5 mM sodium fluoride, protease inhibitor [cOmplete, Mini; Roche Diagnostics GmbH, Mannheim, Germany] and phosphatase inhibitor [PhosSTOP; Roche Diagnostics GmbH]), homogenized for 40 s using FastPrep®-24 Classic homogenizer (MP Biomedicals, Santa Ana, CA, USA), then centrifuged (1000g, 5 min, 4 ºC). Protein concentration of supernatants was measured by colorimetric method with Bradford reagent (Serva Electrophoresis GmbH, Heidelberg, Germany), then samples were diluted to equal (1.5 µg/µL) protein concentration with loading buffer (50 m*M* Tris-hydrochloric acid, 10% glycerol, 2% sodium dodecyl sulphate [Serva Electrophoresis GmbH], 0.1% bromophenol blue and 2% mercaptoethanol; final concentrations) and processed with heat denaturation (5 min, 95 ºC). Electrophoresis was performed in duplicates in 5% stacking (60 V, 30 min) and 8.1% separation (120 V, 90 min) polyacrylamide gel (20 µL loading volume per

lane). After tank blotting (25 V, 20 min), membranes were blocked in 5% bovine serum albumin (BSA) containing phosphate buffered saline with Tween 20 (PBST; 60 min, room temperature), followed by overnight incubation at 4 ºC with primary antibodies (diluted with 5% BSA/PBST) in the following concentrations: 1:500 for GCGR (Santa Cruz Biotechnology, CA, USA); 1:3000 for IRβ (Cell Signaling Technology, Frankfurt, Germany) and 1:2100 for mTOR (Santa Cruz Biotechnology). Detection of primary antibodies was performed using an anti-rabbit secondary antibody coupled with horseradish peroxidase (Cell Signaling Technology), in the concentrations as 1:3000 dilution in 5% BSA/PBST for GCGR, and 1:2500 dilution in 2.5% BSA/PBST for IRβ and mTOR (60 min, room temperature). Finally, chemiluminescence was generated with the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) and detected with ChemiDoc XRS+ system (Bio-Rad Laboratories GmbH.). Bands were quantified with densitometry using Image Lab 4.0 software; relative protein abundance values were gained by standardizing trace quantities to the Indian Ink stained bands.

4.2.3.4. Study III – Weight measurements and chemical analysis of carcass traits

In **Study III**, apart from weighing carcass traits and certain organs, chemical analyses of pectoral and femoral muscle samples were performed at the Institute of Physiology and Nutrition, Hungarian University of Agriculture and Life Sciences (Herceghalom, Hungary) to investigate the possible effects of the dietary factors on the composition of the most valuable meat parts.

Chemical analyses were conducted as outlined by the Association of Analytical Chemists (AOAC 1990). After thawing on ice, dry matter content was measured by drying the samples at 135 ºC for 2 h in line with the appropriate AOAC protocol (method number 930.15). Crude protein content of muscle samples was determined by the Kjeldahl procedure (AOAC method number 920.39); lipid content was assessed as ether extract using a Soxhlet apparatus (AOAC method number 988.05).

4.3. *In vitro* study

For **Study IV** all reagents were purchased from Biolab (Budapest, Hungary), except when otherwise specified. All the incubations were performed at 40 ºC under microaerobic conditions, ensured by Campygen sachets (Oxoid, Basingstoke, UK; catalogue number: CN0035). All methods used in the study were in accordance with the protocols of Clinical and Laboratory Standards Institute documents M31-S1 and M37-E.

4.3.1. *Campylobacter* culturing and determination of bacterial count

C. jejuni strains (7 field isolates and 1 reference strain [ATTC700819]) were provided by the Clinic for Poultry Medicine, University of Veterinary Medicine (Vienna, Austria). Bacteria

were gently thawed from -80 ºC and microorganisms were streaked out onto Campylobacter selective agar (CSA) plates. CSAs were prepared previously by using Campylobacter Agar Base (catalogue number: CAA20500), 5% sterile sheep blood and Campylobacter selective supplement (catalogue number: CCS80004) according to the manufacturer's instructions. These plates were incubated for 48 h. Following the incubation, some colonies were picked up and inoculated into 4 ml Bolton broth (catalogue number: BOB20500), containing Campylobacter selective supplement (catalogue number: CBS80004) as recommended by the producer. *Campylobacter* count of the suspensions was determined after 48 h of culturing, colony-forming unit concentration (CFU/ml) was calculated after further 48 h plating on CSAs by plate counting.

4.3.2. Butyrate treatment of the cultures

Solutions containing different concentrations of sodium butyrate in the range of 5 to 100 mmol/l (5, 7.5, 10, 15, 20, 30, 50, 100 mmol/l) were obtained by dissolving sodium (n)-butyrate in buffered Bolton broth and thereafter performing serial dilution. The pH value of each solution was set at 6.0 or 7.4 by adding the appropriate amount of concentrated hydrochloric acid, to mimic possible *in vivo* cecal pH range evoked by different dietary strategies. All *C. jejuni* strains were tested on all the eight butyrate concentrations listed and at both pH using one positive control free of butyrate and one negative control which did not contain any *Campylobacter* strain. Butyrate dilutions were inoculated with $7*10^5$ CFU/ml *C. jejuni* on 96-well plates in a total volume of 220 µl/well.

4.3.3. Plating and determination of the pH-associated antibacterial efficacy of butyrate

After 48 h incubation of *C. jejuni* strains with different concentrations of butyrate, CFU/ml values were determined by plating in a serial dilution. Each suspension plus controls were diluted (10-fold) up to the 10^{-8} dilutions in phosphate buffered saline (PBS). Afterwards, 100 µl sample was taken out from each dilution and cultivated on CSA. *Campylobacter* colonies were counted after 48 h of culturing; minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined from *Campylobacter* counts. Relative inhibition of *C. jejuni* strains by different concentrations of butyrate were determined as the ratio of CFU/ml values in butyrate-treated wells compared to those of the positive controls (no butyrate). Further, decimal logarithm of relative inhibition was calculated and analyzed in each case. Prediction and confidence intervals were also determined when appropriate.

4.3.4. Antibiotic sensitivity test

Antibiotic sensitivity of the tested strains was assessed with enrofloxacin (5 µg/plate) and ampicillin (10 µg/plate) as well. Tests were performed on CSA plates by conventional agar disk diffusion test. The plates were incubated, thereafter the diameter of the obtained inhibition zone was measured (mm). In case of an inhibition zone below 5 mm diameter, the strain was declared resistant against the antibiotic in question. When testing antibiotics, we did not aim to determine their MIC values, results serve as background data only.

4.4. Statistics

In **Study I** and **III**, statistical processing of data was carried out with R 3.2.2 software. Multivariate analysis of variance (ANOVA analysis) was used to evaluate the main effects of the independent variables (cereal type, dietary crude protein level and butyrate supplementation) on the measured parameters as response (dependent) variables, and in case of any interaction pair wise comparisons of dietary groups were made with post-hoc Tukey-tests. Results of sampling times were analyzed separately, where applicable. Main effects were determined as follows: WB vs. MB diet, LP vs. NP groups and butyrate supplementation vs. no added butyrate. Data were normally distributed and within-group variances were homogenous. Groups receiving maize-based diet with normal protein level, without butyrate completion were used to calculate age-dependent changes (that were independent from all the investigated nutritional factors), by Mann-Whitney test. Results are expressed as means ± standard error of mean (SEM; **Study I**), or as mean ± standard error (SE; **Study III**).

In **Study II**, three independent linear models were fitted to every measured variable, based on the following model:

$$(GCGR, IR\beta, mTOR) \approx Cereal + Protein + Butyrate + Cereal^{*} Protein + Cereal^{*} Butyrate + Protein^{*} Butyrate + Cereal^{*} Protein^{*} Butyrate$$

Model fitting was performed with the *lm* built-in function. Statistical significance of the main and interaction effects was evaluated with multivariate ANOVA analysis, using the Anova function of the *car* package. The R package *emmeans* was used to perform pairwise comparison of the estimated marginal means to unravel the differences behind the different treatment groups. P values and confidence levels were adjusted with the Tukey method.

Results were considered statistically significant when $P < 0.05$ in case of all evaluations.

In **Study IV**, descriptive statistics were performed, due to the limitations of sample size.

The overview of the studies performed in the PhD work can be seen in **Table 8**.

Table 8. Overview of the performed studies

Study No	Type of the Study	Age of the animals (days)	Investigated factor	Investigated parameters
Study I	*in vivo*	7, 21, 42	dietary cereal type dietary CP level unprotected butyrate supplementation	Plasma concentration of TP, albumin, uric acid, glucose, TG, GLP-1, GIP and insulin Plasma activity of AST and CK Body weight of the animals
Study II	*in vivo*	21	dietary cereal type dietary CP level unprotected butyrate supplementation	Hepatic gene expression and protein abundance of GCGR, IRβ and mTOR Body weight of the animals
Study III	*in vivo*	42	dietary CP level unprotected butyrate supplementation protected butyrate supplementation	Weight of carcass traits and chemical analysis of the femoral and pectoral muscles Body weight of the animals
Study IV	*in vitro*	-	unprotected butyrate supplementation pH *C. jejuni* strain-related properties	MIC and MBC values of butyrate Antibiotic resistance of *C. jejuni* strains

CP: Crude protein; TP: Total protein; TG: Triglyceride; GLP-1: Glucagon-like peptide 1; GIP: Glucose-dependent insulinotropic polypeptide; AST: Aspartate aminotransferase; CK: Creatine kinase; GCGR: Glucagon receptor; IRβ: Insulin receptor β subunit; mTOR: Mammalian target of rapamycin; MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration.

5. Results

In this section, only significant main effects – or when relevant, significant interactions – of selected nutritional factors are presented in details.

5.1. Study I, II and III – Body weight results

The body weight and feed intake of the broilers matched or exceeded the standards of the Ross technology in all phases of fattening.

Body weights of animals were higher in groups fed limiting amino acid supplemented LP diet at each measurement ($P = 0.038$ for d 7, $P < 0.001$ for d 21 and $P < 0.001$ for d 42). The WB diet also positively influenced the same parameter at the age of 7 days ($P = 0.007$) and 21 days ($P = 0.001$). Further, protected butyrate (S90, SC40 and SC30) completion elevated the live weight of the birds in all groups compared to control (MB NP Ctr group) on d 21 and 42 ($P < 0.001$ at both time points). The summarized body weight results of all dietary groups are presented in **Table 9**.

Table 9. Body weight data of broiler chickens (**Study I**, **II** and **III**)

Parameter		Abbreviation of dietary group											Significant differences
		MB NP Ctr	**MB NP But**	**MB LP Ctr**	**MB LP But**	**WB NP Ctr**	**WB NP But**	**WB LP Ctr**	**WB LP But**	**MB NP S90**	**MB NP SC40**	**MB NP SC30**	
Body weight (g)	**Day 1**	39.43 ±0.06	39.46 ±0.06	38.97 ±0.06	39.10 ±0.06	38.66 ±0.06	38.72 ±0.05	39.75 ±0.06	39.65 ±0.06	39.09 ±0.69	39.61 ±0.70	39.28 ±0.70	
	Day 7	171.0 ±8.4	179.8 ±4.9	174.9 ±7.1	179.6 ±8.1	177.6 ±9.4	184.7 ±8.4	196.6 ±9.9	211.4 ±9.9	184.3 ±3.3	180.6 ±5.2	177.9 ±4.5	** WB vs. MB * LP vs. NP
	Day 21	679.5 ±20.8	638.1 ±34.1	824.3 ±32.9	864.0 ±28.6	826.0 ±37.2	845.6 ±30.7	821.2 ±34.0	811.6 ±33.7	800.0 ±24.7	794.3 ±22.1	821.5 ±21.3	** WB vs. MB *** LP vs. NP ***S90, SC40, SC30 vs. Ctr
	Day 42	2234.5 ±97.5	2315.6 ±117.8	2934.7 ±47.9	2635.3 ±97.0	2394.0 ±92.2	2406.5 ±112.7	2810.0 ±73.6	2686.5 ±146.4	2738 ±84.3	2719 ±103.5	2700 ±66.4	*** LP vs. NP ***S90, SC40, SC30 vs. Ctr
Average daily body weight gain, day 1-7 (g/day)		21.9	23.4	22.7	23.4	23.2	24.3	26.1	28.6	24.2	23,5	23.1	
Average daily body weight gain, day 7-21 (g/day)		36.3	32.7	46.4	48.9	46.3	47.2	44.6	42.9	44.0	43.8	46.0	
Average daily body weight gain, day 21-42 (g/day)		74.0	79.9	100.5	84.3	74.7	74.3	94.7	89.3	92.3	91.7	89.5	

MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: “Normal protein” group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: “Low protein” group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation; S90: Protected butyrate supplementation of the diet (Intest-Plus S90, 1.0 g/kg diet); SC40: Protected butyrate supplementation of the diet (Intest-Plus SC40, 1.5 g/kg diet); SC30: Protected butyrate supplementation of the diet (Intest-Plus SC30, 2.0 g/kg diet). Results are expressed as mean ± standard error of mean (SEM). Statistical analysis of data was performed with multi-way ANOVA test to evaluate main effects and by post hoc tests to assess pairwise interactions (S90, SC40, SC30 vs. MB NP Ctr). Main effects were determined as follows: WB vs. MB diet, LP vs. NP groups and butyrate supplementation vs. no added butyrate. n = 10 per sampling points/group, n = 30 in total/group. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

5.2. Study I – General metabolic responsiveness of chickens to nutritional factors

WB diet increased ($P < 0.001$), but as an opposite, LP diet decreased ($P < 0.001$) the total protein (TP) level of blood plasma of chickens at the age of 21 days (**Table 10**).

In order to monitor relative changes of albumin levels, albumin concentration data were used to calculate albumin/TP ratio, which was decreased by butyrate supplementation ($P = 0.022$) on d 7 (**Table 10**).

Concentration of uric acid was significantly elevated in WB groups on d 7 ($P = 0.022$) and d 21 ($P < 0.001$). However, decreased uric acid levels were measured in animals fed LP diet on d 21 ($P < 0.001$) and 6 ($P = 0.002$), while butyrate supplementation increased the same parameter on d 21 only ($P = 0.048$). Plasma uric acid levels gradually decreased by nearly 50% during the experimental period, irrespective of dietary treatment ($P < 0.001$; **Table 10**).

Aspartate aminotransferase activity of chickens was stimulated by WB diet only at the age of 21 days ($P = 0.042$; **Table 10**).

Plasma creatine kinase activity was elevated by lowered crude protein level on d 21 ($P = 0.004$) and 6 ($P = 0.041$), respectively. In addition, enzyme activity increased by nearly thirteen times until d 42, compared to d 7 ($P < 0.001$), independently from diet composition (**Table 10**).

Table 10. Results of the parameters describing metabolism of nitrogen containing compounds (**Study I**)

		Abbreviation of dietary group								
Parameter	**Days**	**MB NP Ctr**	**MB NP But**	**MB LP Ctr**	**MB LP But**	**WB NP Ctr**	**WB NP But**	**WB LP Ctr**	**WB LP But**	**Significant differences**
Total protein (g/l)	7	22.58 ±1.47	22.90 ±1.42	23.03 ±1.57	23.36 ±1.01	26.34 ±1.51	23.99 ±1.07	24.08 ±1.41	23.24 ±1.25	-
	21	24.58 ±0.84	26.08 ±0.87	24.65 ±0.80	23.48 ±0.75	28.99 ±1.05	29.53 ±0.71	25.80 ±1.48	25.10 ±0.58	*** WB vs. MB *** LP vs. NP
	42	25.12 ±1.36	27.30 ±1.51	27.61 ±0.94	27.46 ±1.21	29.01 ±1.23	27.27 ±1.00	29.79 ±0.95	27.21 ±1.14	-
Albumin/ TP (%)	7	55.92 ±1.74	53.21 ±1.88	55.20 ±1.95	53.90 ±2.18	54.96 ±1.86	51.23 ±1.01	54.48 ±1.65	51.19 ±1.82	* But vs. Ctr
	21	48.00 ±0.97	44.95 ±1.29	46.34 ±1.12	45.68 ±0.57	45.17 ±2.19	45.12 ±1.56	45.25 ±1.88	46.62 ±1.25	-
	42	49.21 ±1.40	47.88 ±1.58	47.78 ±1.71	48.73 ±1.96	48.17 ±1.68	47.92 ±2.51	48.76 ±2.34	49.59 ±1.90	-
Uric acid (micromol/l)	7	418.7 ±36.1	350.1 ±26.1	369.6 ±30.4	387.7 ±28.8	478.4 ±44.7	412.2 ±25.2	412.5 ±32.3	443.9 ±39.6	* WB vs. MB
	21	269.6 ±28.7	310.5 ±20.5	188.3 ±16.6	216.2 ±24.4	308.9 ±23.4	355.9 ±24.9	298.3 ±27.1	312.9 ±19.5	*** WB vs. MB *** LP vs. NP * But vs. Ctr
	42	194.3 ±15.3	190.8 ±16.8	135.4 ±17.2	182.9 ±13.3	215.0 ±22.3	227.1 ±14.9	160.8 ±11.1	192.0 ±21.0	** LP vs. NP
Aspartate amino-transferase (IU/l)	7	178.8 ±16.6	168.3 ±10.7	183.6 ±12.9	170.6 ±7.1	198.0 ±18.2	174.4 ±9.8	191.4 ±10.3	174.9 ±7.6	-
	21	160.6 ±20.4	165.8 ±5.7	150.1 ±5.4	157.4 ±7.4	164.9 ±9.3	172.4 ±7.4	174.9 ±12.4	185.4 ±14.9	* WB vs. MB
	42	279.9 ±42.1	337.8 ±39.3	339.9 ±21.8	264.6 ±25.9	288.3 ±33.2	237.6 ±24.7	333.9 ±33.6	333.6 ±24.9	-
Creatine kinase (IU/l)	7	1220 ±94	1590 ±287	1541 ±194	1430 ±161	1148 ±87	1330 ±254	1494 ±94	1220 ±92	-
	21	1374 ±160	1142 ±92	1408 ±251	2163 ±351	1437 ±196	1238 ±109	1994 ±189	1436 ±221	** LP vs. NP
	42	1552 9±47 70	2773 5±84 71	2208 7±30 70	1487 7±31 87	1375 3±42 97	9733 ±277 8	2972 9±62 38	3064 6±59 77	* LP vs. NP

MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium (n-)butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Diet with no sodium (n-)butyrate supplementation; TP: Total protein. Results are expressed as mean ± standard error of mean (SEM). Statistical analysis of data was performed with multi-way ANOVA test to evaluate main effects. Main effects were determined as follows: WB vs. MB diet, LP vs. NP groups and butyrate supplementation vs. no added butyrate.

n = 10 per sampling points per group, n = 30 in total per group.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

Blood glucose level of animals decreased in WB groups at the age of 21 days ($P = 0.002$; **Table 11**).

Significantly higher amounts of circulating triglyceride were measured in groups fed WB diet on d 21 ($P = 0.011$; **Table 11**).

Table 11. Results of glucose and triglyceride measurements (**Study I**)

		Abbreviation of dietary group								
Parameter	**Days**	**MB NP Ctr**	**MB NP But**	**MB LP Ctr**	**MB LP But**	**WB NP Ctr**	**WB NP But**	**WB LP Ctr**	**WB LP But**	**Significant differences**
Glucose (mmol/l)	7	14.07 ±1.34	11.90 ±0.37	18.58 ±2.03	13.28 ±0.51	12.64 ±0.71	18.69 ±2.14	15.95 ±1.72	13.91 ±1.01	-
	21	15.69 ±1.60	16.81 ±1.71	15.84 ±1.61	15.10 ±1.64	13.34 ±0.53	13.16 ±0.39	13.38 ±0.38	13.23 ±0.40	** WB vs. MB
	42	14.83 ±0.35	14.65 ±0.32	13.81 ±0.38	14.08 ±0.43	14.29 ±0.37	13.50 ±0.44	14.39 ±0.35	14.45 ±0.36	-
Triglyceride (mmol/l)	7	0.752 ±0.047	0.634 ±0.062	0.648 ±0.034	0.738 ±0.057	0.821 ±0.037	0.681 ±0.039	0.746 ±0.077	0.714 ±0.061	-
	21	0.542 ±0.071	0.557 ±0.053	0.836 ±0.073	0.774 ±0.112	0.837 ±0.067	0.866 ±0.090	0.666 ±0.088	0.934 ±0.066	* WB vs. MB
	42	0.792 ±0.076	1.006 ±0.094	0.995 ±0.169	0.995 ±0.110	1.313 ±0.100	1.138 ±0.066	0.951 ±0.131	1.009 ±0.089	-

MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: “Normal protein” group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: “Low protein” group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium (n-)butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Diet with no sodium (n-)butyrate supplementation. Results are expressed as mean ± standard error of mean (SEM). Statistical analysis of data was performed with multi-way ANOVA test to evaluate main effects. Main effects were determined as follows: WB vs. MB diet, LP vs. NP groups and butyrate supplementation vs. no added butyrate.

n = 10 per sampling points per group, n = 30 in total per group.

** $P < 0.01$; * $P < 0.05$

In case of GLP-1, GIP and insulin, plasma levels seemed not respond to any of the investigated nutritional factors, although we detected an increase by 100 % for GLP-1 ($P = 0.005$) and for insulin ($P < 0.001$) on d 21, as well as an increase by 60% for GLP-1 ($P = 0.007$), and by 30% for insulin ($P < 0.001$) on d 42, compared to d 7 measurements (**Table 12**).

Table 12. Results of the parameters describing insulin homeostasis (**Study I**)

		Abbreviation of dietary group								
Parameter	**Days**	**MB NP Ctr**	**MB NP But**	**MB LP Ctr**	**MB LP But**	**WB NP Ctr**	**WB NP But**	**WB LP Ctr**	**WB LP But**	**Significant differences**
GLP-1 (pg/ml)	7	148.1 ±21.9	101.9 ±16.6	76.1 ±7.4	82.2 ±8.3	119.1 ±14.9	122.2 ±16.6	124.4 ±13.7	139.1 ±10.4	-
	21	290.3 ±31.8	292.0 ±20.4	292.1 ±15.7	276.5 ±23.1	246.5 ±12.9	266.4 ±22.3	273.9 ±16.0	274.9 ±14.7	-
	42	240.5 ±11.8	218.6 ±10.3	218.0 ±11.3	219.6 ±10.0	218.3 ±12.6	233.6 ±10.3	215.4 ±7.4	265.0 ±18.5	-
GIP (pg/ml)	7	147.9 ±32.8	143.4 ±46.2	126.9 ±60.8	123.9 ±36.3	153.1 ±43.7	102.9 ±40.2	260.8 ±97.2	172.4 ±42.8	-
	21	181.0 ±43.9	188.7 ±48.1	178.8 ±43.3	242.9 ±66.8	118.9 ±40.9	112.9 ±53.7	163.1 ±56.4	144.3 ±39.6	-
	42	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-
Insulin (ng/ml)	7	4.071 ±0.057	4.148 ±0.126	3.811 ±0.026	3.976 ±0.064	3.926 ±0.108	3.831 ±0.046	4.132 ±0.171	3.931 ±0.047	-
	21	8.377 ±0.290	9.099 ±0.465	8.568 ±0.421	8.857 ±0.240	9.169 ±0.554	9.285 ±0.714	9.123 ±0.630	8.237 ±0.241	-
	42	5.209 ±0.283	5.527 ±.413	6.081 ±0.504	5.401 ±0.450	5.688 ±0.272	5.383 ±0.340	4.783 ±0.175	5.403 ±0.326	-

MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium (n-)butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Diet with no sodium (n-)butyrate supplementation. GLP-1: Glucagon-like peptide 1; GIP: Glucose-dependent insulinotropic polypeptide. Results are expressed as mean ± standard error of mean (SEM). Statistical analysis of data was performed with multi-way ANOVA test to evaluate main effects. Main effects were determined as follows: WB vs. MB diet, LP vs. NP groups and butyrate supplementation vs. no added butyrate.
n = 10 per sampling points per group, n = 30 in total per group.

5.3. Study II – Changes in hepatic insulin and glucagon signaling

In this section, only significant main and interaction effects of the investigated dietary factors are presented in details.

5.3.1. Glucagon receptor

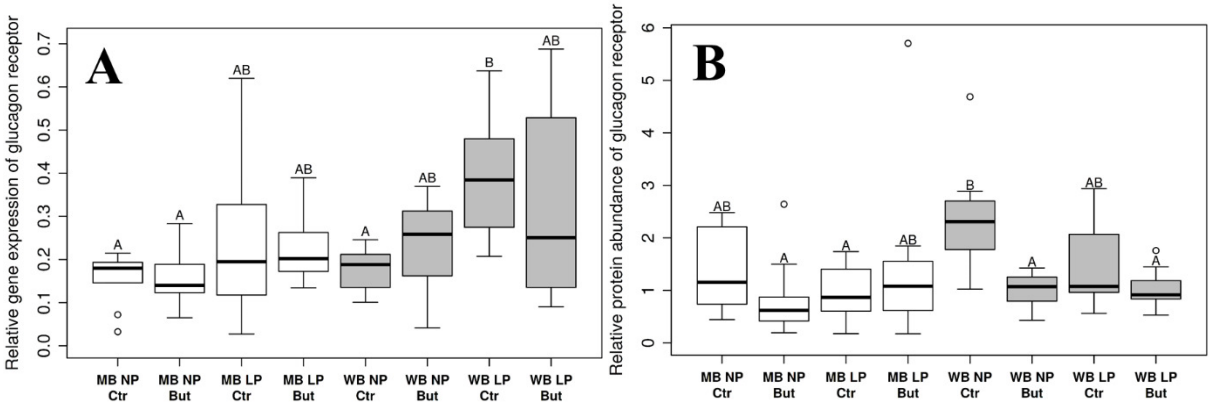
Diet-evoked changes in GCGR gene expression together with the results of pairwise comparisons can be seen in **Figure 2A**.

Multivariate ANOVA analysis showed that GCGR gene expression increased in WB compared to MB groups ($P < 0.001$) and in chickens kept on LP diet in contrast to animals of the NP groups ($P = 0.0067$; for more details, see **Table S1** and **S2**). Pairwise comparisons revealed significant differences between WB LP Ctr vs. MB NP Ctr ($P = 0.0083$), MB NP But ($P = 0.0150$) and WB NP Ctr groups ($P = 0.0221$; more details can be seen in **Table S3**).

Diet-related changes in GCGR protein abundance together with the results of pairwise comparisons can be seen in **Figure 2B**.

On the protein level, decreasing effect of butyrate (P = 0.0224) and the interactions of the dietary cereal type and butyrate supplementation (P = 0.0226), as well as crude protein content and butyrate supplementation (P = 0.0172) were observed (further details: **Table S1** and **S2**). Pairwise comparisons showed significant differences between WB NP Ctr vs. MB LP Ctr (P = 0.0157), MB NP But (P = 0.0057), WB LP But (P = 0.0221) and WB NP But groups (P = 0.0212; for further details, see **Table S4**.

Figure 2. Relative gene expression (A) and protein abundance (B) of hepatic glucagon receptor (**Study II**)



MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation. Letters indicate groups that were significantly different with pairwise comparison, if two groups have different letters, that means P values were < 0.05.
Statistical analysis of data was performed with multivariate ANOVA test to evaluate main effects, pairwise comparisons were implemented by the *emmeans* R package with P value adjustments with the Tukey method. Empty boxes refer to MB, grey boxes refer to WB groups.
n = 10 per group.

5.3.2. Insulin receptor β

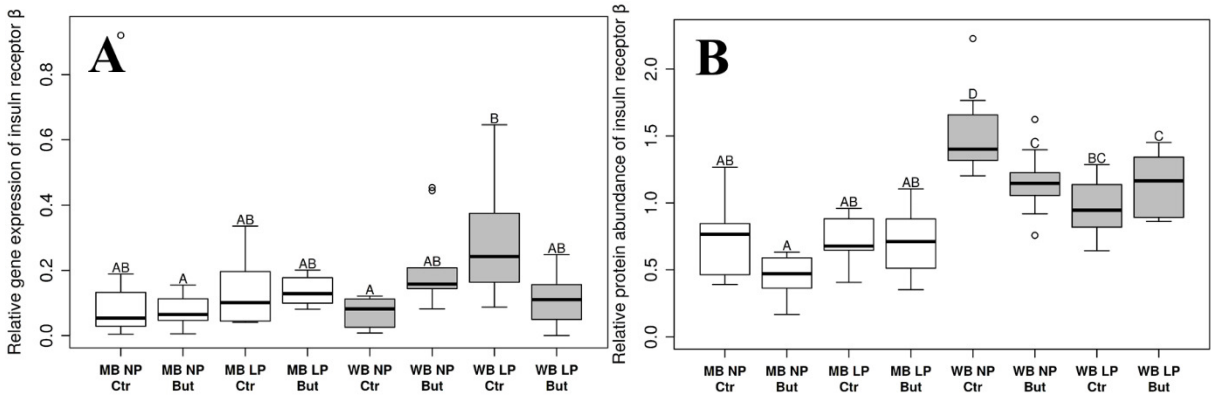
Diet-induced changes in IRβ gene expression together with the results of pairwise comparisons can be seen in **Figure 3A**.

Significant three-way interaction effect between all independent variables was detected with ANOVA analysis in case of IRβ mRNA concentration (P = 0.0028; **Table S1** and **S2**). Pairwise comparisons revealed differences between WB LP Ctr vs. WB NP Ctr (P = 0.0226) and MB NP But groups (P = 0.0270; **Table S5** shows additional details on the pairwise comparisons.

Diet-evoked changes in IRβ protein abundance together with the results of pairwise comparisons can be seen in **Figure 3B**.

Lowering effect of butyrate (P = 0.0343) and augmenting effect of WB diet (P < 0.001) were detected as main effects, further, significant cereal:protein (P < 0.001) and protein:butyrate (P < 0.001) interactions were found by the ANOVA analysis of the IRβ western blot results, referring to protein abundance (more details can be seen in **Table S1** and **S2**). According to the pairwise comparisons, either WB LP But, WB NP But and WB NP Ctr groups differed significantly from every MB groups (P values ranging from < 0.001 to 0.0132), while WB LP Ctr was only different from the MB NP But treatment from the MB group (P < 0.001). Considering the WB group solely, WB NP Ctr was different from every other WB treatment (P values ranging from < 0.001 to 0.0417), while no significant differences were detected in the MB groups (for more details, see **Table S6**).

Figure 3. Relative gene expression (A) and protein abundance (B) of hepatic insulin receptor β (**Study II**)



MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation. Letters indicate groups that were significantly different with pairwise comparison, if two groups have different letters, that means P values were < 0.05.
Statistical analysis of data was performed with multivariate ANOVA test to evaluate main effects, pairwise comparisons were implemented by the *emmeans* R package with P value adjustments with the Tukey method. Empty boxes refer to MB, grey boxes refer to WB groups.
n = 10 per group.

5.3.3. Mammalian target of rapamycin

Diet-related changes in mTOR gene expression together with the results of pairwise comparisons can be seen in **Figure 4A**.

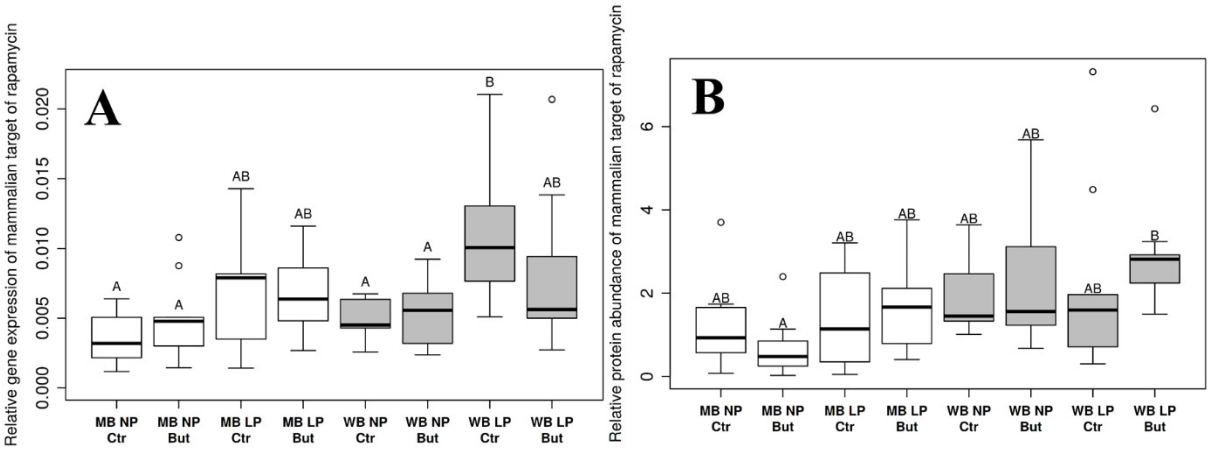
The mTOR mRNA concentration showed an increase in chickens fed WB diet in comparison with their counterparts reared on MB diet (P = 0.0456) and was augmented in LP group compared to animals fed NP diet (P < 0.001; for more details: **Table S1** and **S2**). Further,

significant differences were found between the WB LP Ctr vs. MB NP But (P = 0.0131), MB NP Ctr (P = 0.0012), WB NP But (P = 0.0179) and WB NP Ctr treatment groups (P = 0.0094; **Table S7**).

Diet-induced hanges in mTOR protein abundance together with the results of pairwise comparisons can be seen in **Figure 4B**.

Protein abundance of mTOR was higher in animals fed WB diet than in those kept on MB diet (P = 0.001). Considering the pairwise comparisons, only the MB NP But and WB LP But groups differed significantly (P = 0.0199; more details can be seen in **Table S1**, **S2** and **S8**).

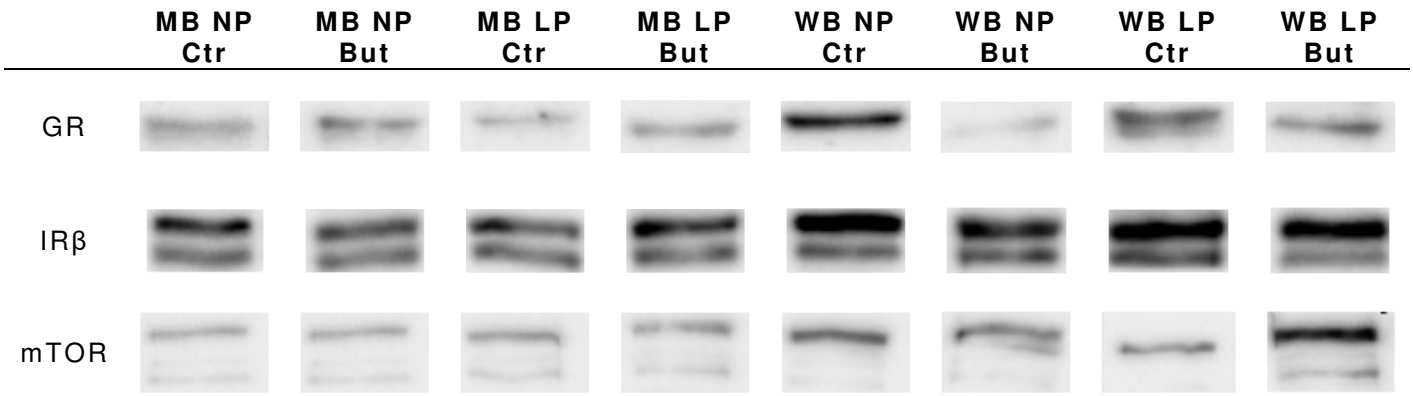
Figure 4. Relative gene expression (A) and protein abundance (B) of hepatic mammalian target of rapamycin (**Study II**)



MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation. Letters indicate groups that were significantly different with pairwise comparison, if two groups have different letters, that means P values were < 0.05.
Statistical analysis of data was performed with multivariate ANOVA test to evaluate main effects, pairwise comparisons were implemented by the *emmeans* R package with P value adjustments with the Tukey method. Empty bars refer to MB, grey bars refer to WB groups.
n = 10 per group.

Representative bands of the signaling elements obtained by Western blotting are presented in **Table 13.**

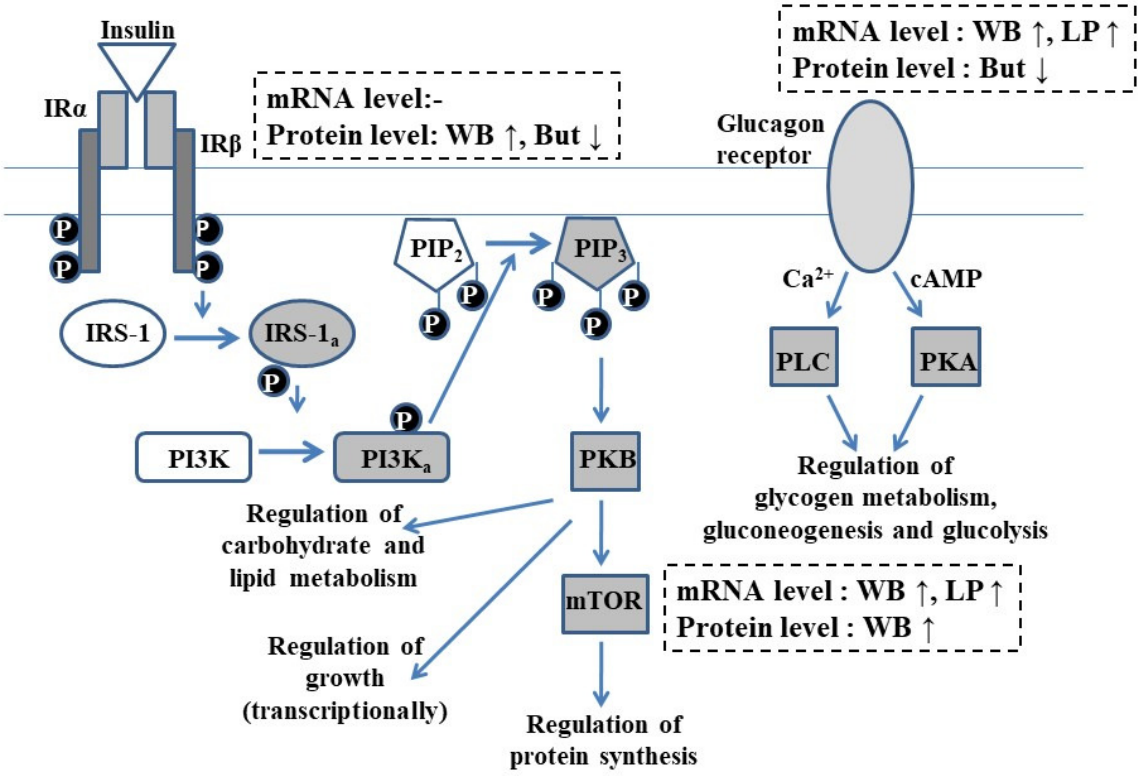
Table 13. Representative bands corresponding to the investigated proteins as obtained by Western blotting (**Study II**)



GCGR: Glucagon receptor (57 kDal); IRβ: Insulin receptor β subunit (95 kDal); mTOR: Mammalian target of rapamycin (289 kDal). MB: Maize based diet; WB: Wheat based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation.

The observed diet-associated main effects on insulin and glucagon signaling are summarized in **Figure 5**, also providing an overview of the signaling pathways.

Figure 5. Overview of the insulin and glucagon signaling pathways and the observed main effects of the investigated nutritional factors (**Study II**)



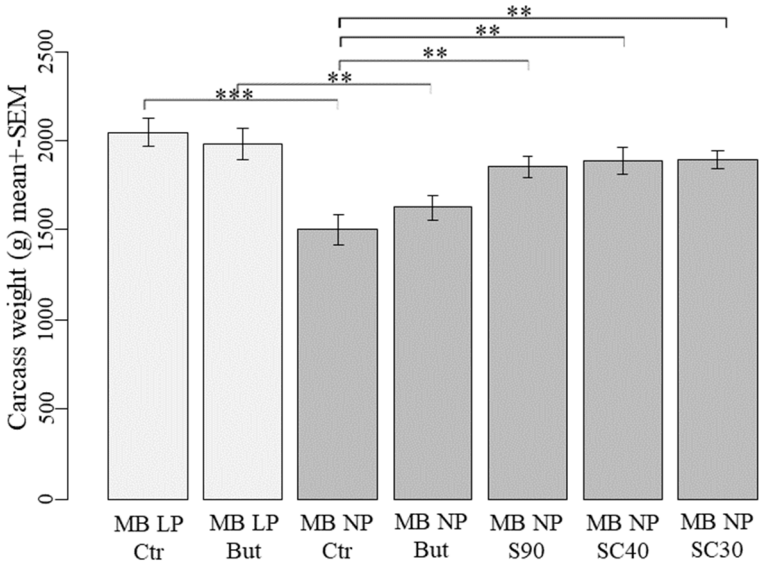
IRα: Insulin receptor α subunit; IRβ: Insulin receptor β subunit; IRS-1: Insulin receptor substrate 1 ("a" lowercase subscript when activated); PI3K: Phosphatidylinositol-3-kinase ("a" lowercase subscript when activated); PIP_2: Phosphatidylinositol diphosphate; PIP_3: Phosphatidylinositol triphosphate; PKB: Protein kinase B; mTOR: Mammalian target of rapamycin; Ca^{2+}: Calcium cation; cAMP: cyclic adenosine monophosphate; PLC: Phospholipase C; PKA: Protein kinase A; P: phosphate group; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet). ↓ and ↑ arrows indicate the lowering or increasing effect of the given nutritional factor on the investigated parameter. The figure was created by the author.

5.4. Study III – Nutritional effects on the weights of organs and carcass characteristics

Carcass weight (**Figure 6**) significantly elevated as the effect of low-protein diet with essential amino acid completion: significantly higher values were measured in the LP Ctr than in NP Ctr group ($P < 0.001$) and in the LP But than in the NP But group ($P = 0.005$). No significant difference of unprotected sodium (n)-butyrate supplementation was observed compared to Ctr groups. However, all protected sodium butyrate products (fed in NP S90, NP SC40 and NP

SC30 groups) proved to increase carcass weight, compared to control (NP Ctr group; P = 0.009, P = 0.003 and P = 0.002, respectively).

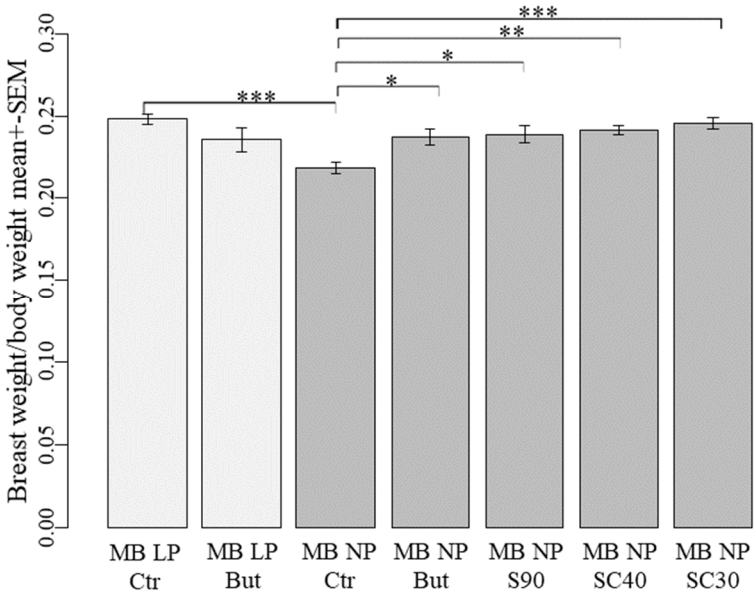
Figure 6. Carcass weight (**Study III**)



MB: Maize-based diet; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation; S90: Protected butyrate supplementation of the diet (Intest-Plus S90, 1.0 g/kg diet); SC40: Protected butyrate supplementation of the diet (Intest-Plus SC40, 1.5 g/kg diet); SC30: Protected butyrate supplementation of the diet (Intest-Plus SC30, 2.0 g/kg diet). Results are expressed as mean ± standard error (SE). Statistical analysis of data was performed with multi-way ANOVA test to evaluate main effects and by post hoc tests. Light bars refer to LP, dark bars refer to NP groups.
n = 10 per per group.
Significant differences revealed by post hoc tests are marked as follows:
*** P < 0.001; ** P < 0.01

The relative weight of deboned breast meat (**Figure 7**) was greater in the LP Ctr than in the NP Ctr group (P < 0.001). Additionally, all forms of butyrate significantly (NP But: P = 0.046; NP S90: P = 0.003; NP SC40: P = 0.007; and NP SC30: P < 0.001) elevated breast meat yield when compared to control (NP Ctr group), but no such stimulatory effect of unprotected butyrate was found in case of LP groups.

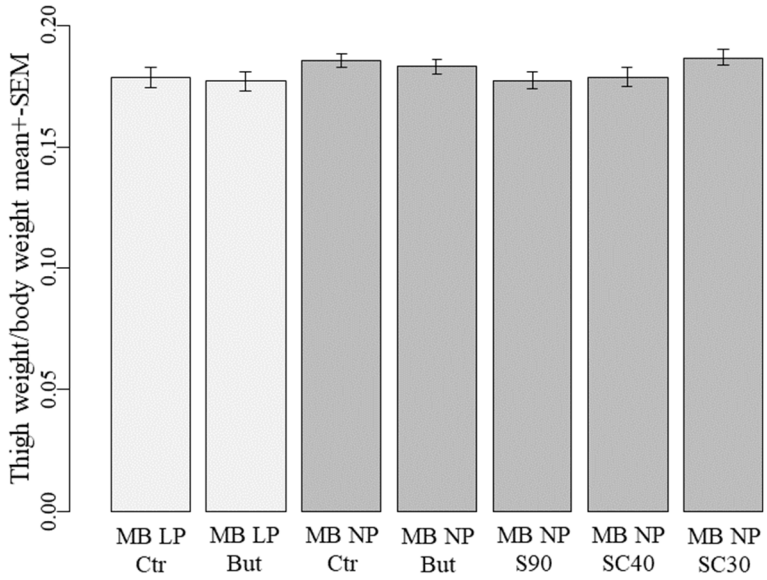
Figure 7. Relative deboned breast meat yield (**Study III**)



MB: Maize-based diet; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation; S90: Protected butyrate supplementation of the diet (Intest-Plus S90, 1.0 g/kg diet); SC40: Protected butyrate supplementation of the diet (Intest-Plus SC40, 1.5 g/kg diet); SC30: Protected butyrate supplementation of the diet (Intest-Plus SC30, 2.0 g/kg diet). Results are expressed as mean ± standard error (SE). Statistical analysis of data was performed with multi-way ANOVA test to evaluate main effects and by post hoc tests. Light bars refer to LP, dark bars refer to NP groups.
n = 10 per per group.
Significant differences revealed by post hoc tests are marked as follows:
*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

In contrast to the breast meat, no significant differences were observed regarding the relative mass of thighs (**Figure 8**) between any experimental groups.

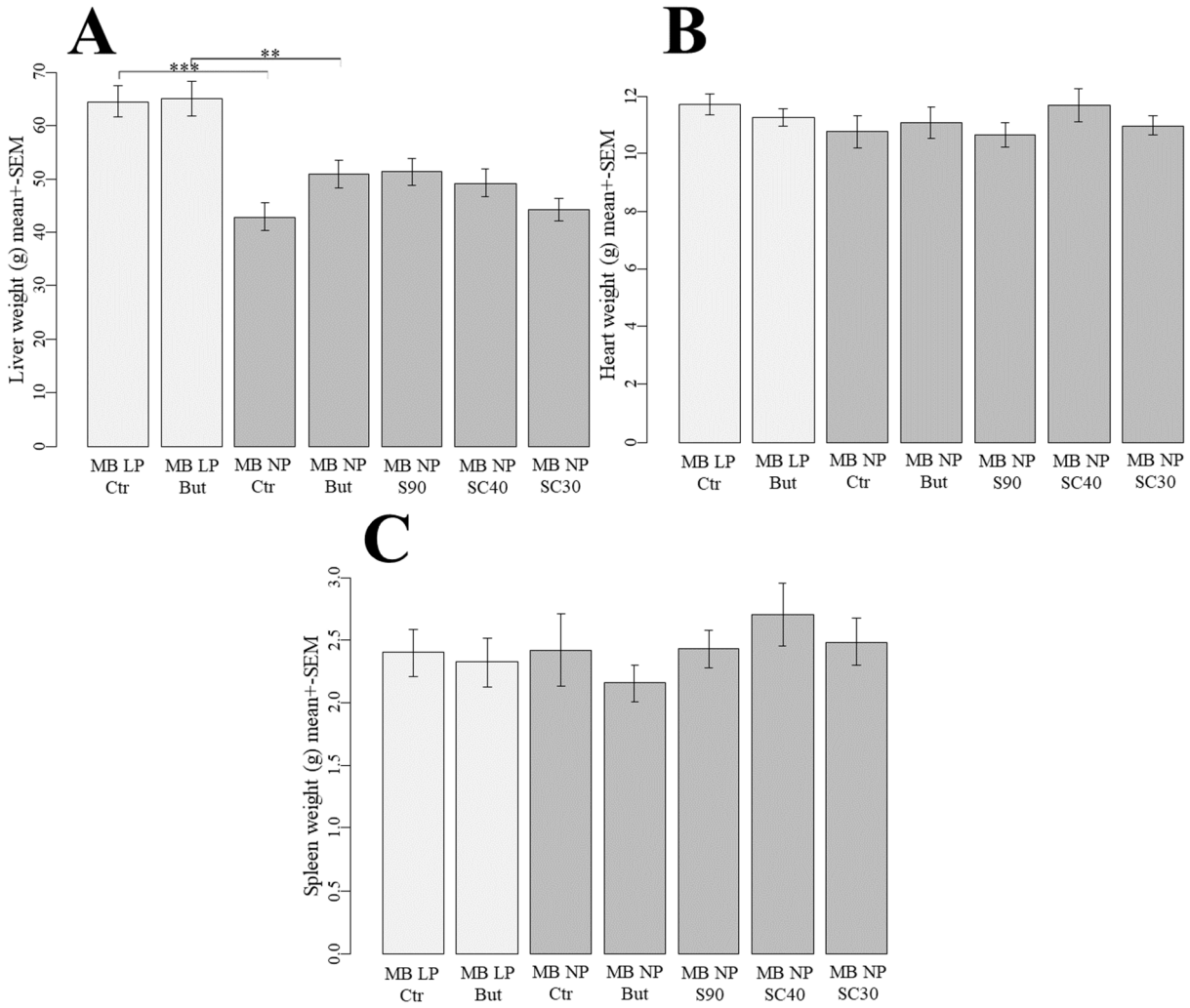
Figure 8. Relative thigh yield (**Study III**)



MB: Maize-based diet; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation; S90: Protected butyrate supplementation of the diet (Intest-Plus S90, 1.0 g/kg diet); SC40: Protected butyrate supplementation of the diet (Intest-Plus SC40, 1.5 g/kg diet); SC30: Protected butyrate supplementation of the diet (Intest-Plus SC30, 2.0 g/kg diet). Results are expressed as mean ± standard error (SE). Statistical analysis of data was performed with multi-way ANOVA test to evaluate main effects and by post hoc tests. Light bars refer to LP, dark bars refer to NP groups.
n = 10 per per group.

Liver weight (**Figure 9A**) was greater in LP Ctr than in NP Ctr animals ($P < 0.001$) and in LP But than in NP But chickens ($P = 0.003$). In the case of further giblets (heart and spleen; **Figure 9B** and **9C**), no significant differences were detected.

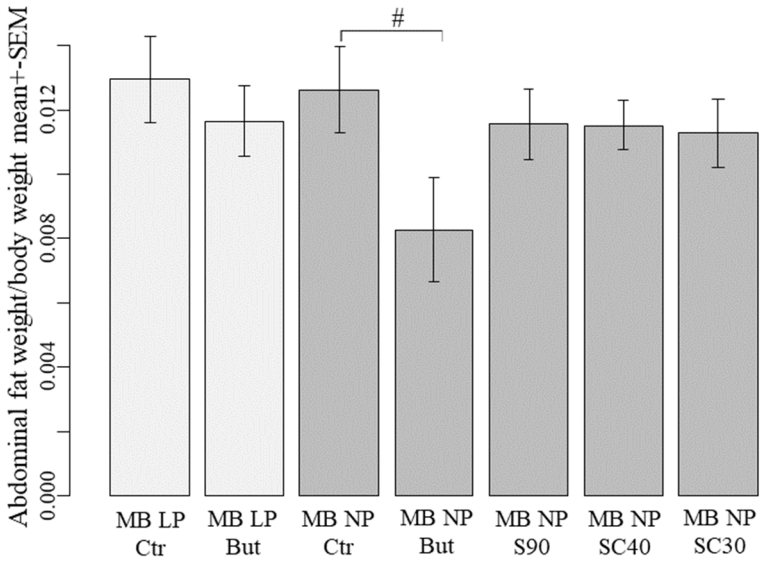
Figure 9. Weight of liver (A), heart (B) and spleen (C; **Study III**)



MB: Maize-based diet; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation; S90: Protected butyrate supplementation of the diet (Intest-Plus S90, 1.0 g/kg diet); SC40: Protected butyrate supplementation of the diet (Intest-Plus SC40, 1.5 g/kg diet); SC30: Protected butyrate supplementation of the diet (Intest-Plus SC30, 2.0 g/kg diet). Results are expressed as mean ± standard error (SE). Statistical analysis of data was performed with multi-way ANOVA test to evaluate main effects and by post hoc tests. Light bars refer to LP, dark bars refer to NP groups.
n = 10 per per group.
Significant differences revealed by post hoc tests are marked as follows:
*** $P < 0.001$; ** $P < 0.01$

Relative abdominal fat mass (**Figure 10**) tended to be decreased by unprotected sodium butyrate (NP But compared to the NP Ctr group: $P = 0.077$), but no significant effects were found with regard to dietary crude protein levels or protected sodium butyrate products.

Figure 10. Relative abdominal fat weight (**Study III**)



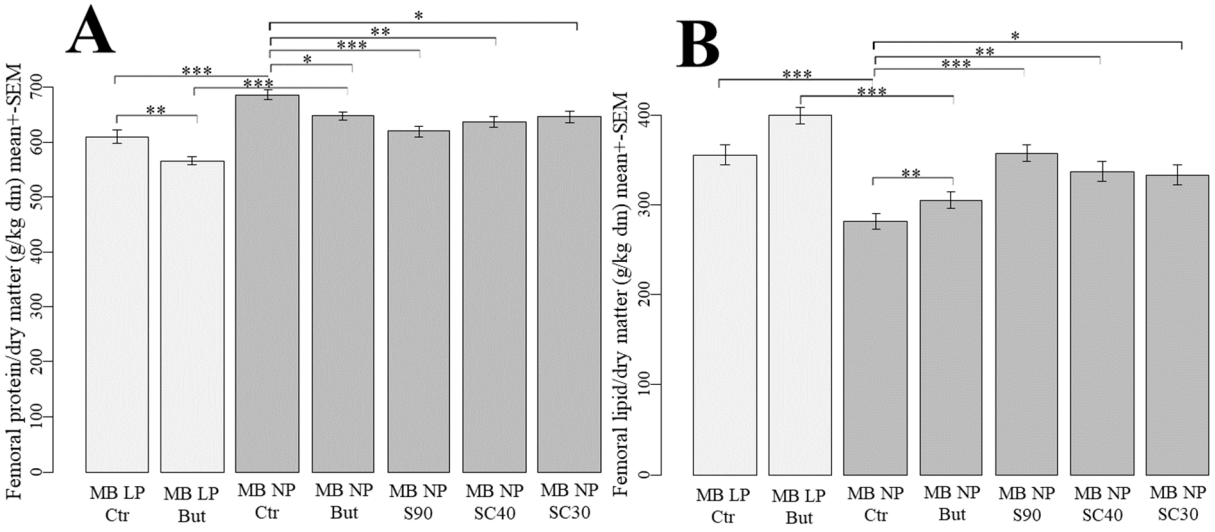
MB: Maize-based diet; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation; S90: Protected butyrate supplementation of the diet (Intest-Plus S90, 1.0 g/kg diet); SC40: Protected butyrate supplementation of the diet (Intest-Plus SC40, 1.5 g/kg diet); SC30: Protected butyrate supplementation of the diet (Intest-Plus SC30, 2.0 g/kg diet). Results are expressed as mean ± standard error (SE). Statistical analysis of data was performed with multi-way ANOVA test to evaluate main effects and by post hoc tests. Light bars refer to LP, dark bars refer to NP groups.
n = 10 per per group.
Tendention revealed by post hoc test is marked as follows:
$^{\#}$ $P < 0.10$

The chemical analysis of muscle composition revealed that LP diet and all forms of butyrate decreased the protein content of the femoral muscle (**Figure 11A**). In details, lowered protein content was measured in the LP Ctr than the NP Ctr group ($P < 0.001$) and, similarly, in the thighs of LP But chicks compared to those of NP But animals ($P < 0.001$). The lowering effect of unprotected sodium butyrate could be observed between NP But and NP Ctr groups ($P = 0.031$) and between LP But and LP Ctr groups ($P = 0.008$) as well. A significant reduction in femoral protein content was also measured in the case of all types of protected sodium butyrate applied (NP S90: $P < 0.001$; NP SC40: $P = 0.002$; and NP SC30: $P = 0.02$) when compared to control animals (NP Ctr group).

The lipid content of the femoral muscle (**Figure 11B**) was affected by the dietary crude protein level and by butyrate supplementation as well. Significantly higher values were measured in the thighs of chickens kept on a low-protein, amino-acid-completed diet than in those of the NP groups (LP Ctr compared to NP Ctr group: $P < 0.001$; LP But compared to NP But animals: $P < 0.001$). All types of sodium butyrate supplementation elevated the lipid content

of the femoral muscle (NP But: $P = 0.018$; NP S90: $P < 0.001$; NP SC40: $P = 0.001$; and NP SC30: $P = 0.003$) when compared to butyrate-free control (NP Ctr group).

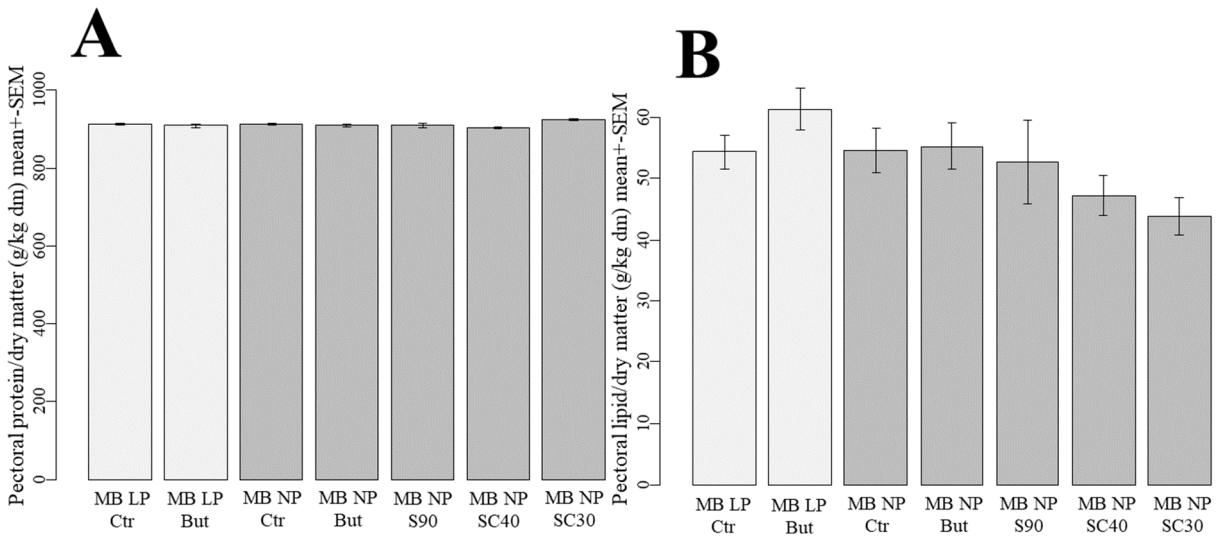
Figure 11. Protein (A) and lipid (B) content of the femoral muscle (**Study III**)



MB: Maize-based diet; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation; S90: Protected butyrate supplementation of the diet (Intest-Plus S90, 1.0 g/kg diet); SC40: Protected butyrate supplementation of the diet (Intest-Plus SC40, 1.5 g/kg diet); SC30: Protected butyrate supplementation of the diet (Intest-Plus SC30, 2.0 g/kg diet). Results are expressed as mean ± standard error (SE). Statistical analysis of data was performed with multi-way ANOVA test to evaluate main effects and by post hoc tests. Light bars refer to LP, dark bars refer to NP groups.
n = 10 per per group.
Significant differences revealed by post hoc tests are marked as follows:
*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

The protein (**Figure 12A**) and lipid (**Figure 12B**) content of pectoral muscle remained unchanged by any of the dietary treatments.

Figure 12. Protein (A) and lipid (B) content of the pectoral muscle (**Study III**)



MB: Maize-based diet; NP: "Normal protein" group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: "Low protein" group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation; S90: Protected butyrate supplementation of the diet (Intest-Plus S90, 1.0 g/kg diet); SC40: Protected butyrate supplementation of the diet (Intest-Plus SC40, 1.5 g/kg diet); SC30: Protected butyrate supplementation of the diet (Intest-Plus SC30, 2.0 g/kg diet). Results are expressed as mean ± standard error (SE). Statistical analysis of data was performed with multi-way ANOVA test to evaluate main effects and by post hoc tests. Light bars refer to LP, dark bars refer to NP groups.
n = 10 per per group.

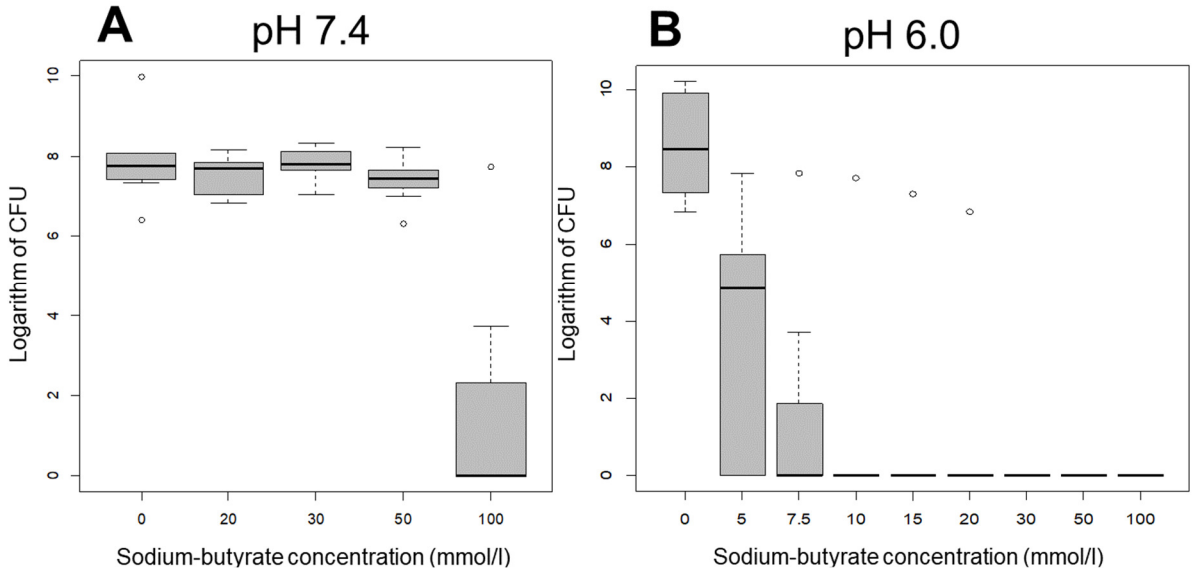
5.5. Study IV – The *in vitro* antibacterial efficacy of butyrate and antibiotic sensitivity of *C. jejuni* strains

5.5.1. The *in vitro* pH-associated antibacterial efficacy of butyrate

It could be observed that incubation of bacteria in Bolton broth on 96-well-plates for 48 h caused approx. 1000-fold increase in CFU compared to the inoculated count of bacteria ($7*10^5$ CFU/ml) in positive controls. In order to reach the MIC value, a decimal logarithm of relative inhibition of -3 had to be gained to inhibit growth of the inoculated bacteria. According to its definition, MBC refers to the concentration of an agent that kills 99.9% of the bacteria, thus, a decimal logarithm of relative inhibition of -6 matched the requirements of MBC, when only 0.1% of the MIC bacteria count could survive.

The decimal logarithm of the calculated CFU/ml values are plotted out in **Figure 13.** Butyrate showed a remarkable inhibitory effect on *C. jejuni* strains in the established *in vitro* model. This effect was found to be highly pH-dependent, MIC and MBC values decreased intensively at pH 6.0 (**Figure 13A**), compared to those measured at pH 7.4 (**Figure 13B**).

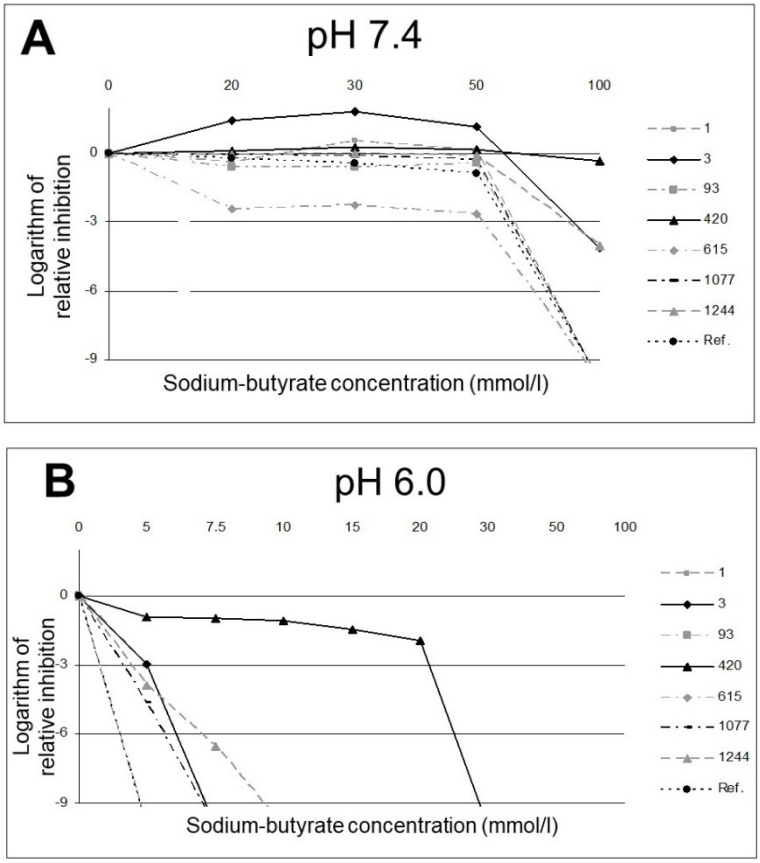
Figure 13. Decimal logarithm of CFU/ml values of *C. jejuni* strains per ml broth in the presence of various concentrations of butyrate at pH 7.4 (A) and 6.0 (B; **Study IV**)



Medians, upper and lower quartiles, maximum and minimum values and outliers are shown in boxplots.

When pH of incubation was set at 7.4, MIC value revealed to be 100 mmol/l butyrate for all strains except one (No 420). The applied 100 mmol/l concentration met the requirements of MBC as well in case of 5 strains because of the intensive decrease in *Campylobacter* counts (**Figure 14A**). At pH 6.0, antibacterial efficacy of butyrate proved to highly increase, already 5 mmol/l butyrate inhibited growth of *Campylobacter*s (MIC), and MBC was observed as well at 5 or 7.5 mmol/l concentrations depending on the strains. Interestingly, similarly to what observed at higher pH, strain No 420 seemed to be strongly resistant against butyrate at pH 6.0 as well (**Figure 14B**).

Figure 14. Decimal logarithm of relative inhibition of different butyrate concentrations on *C. jejuni* strains at pH 7.4 (A) and 6.0 (B; **Study IV**)



Relative inhibition was considered as the ratio of CFU values in butyrate-treated wells compared to those of the positive controls (no butyrate). Field isolate strains are marked with numbers, while “Ref.” refers to the tested reference strain.

Prediction and confidence intervals of 90% was also determined for MIC values at pH 6.0. According to our results, the predicted value of MIC can be considered as 3.87 mM butyrate with a 90% prediction interval of 0 – 8.01 mmol/l and 90% confidence interval of 2.75 – 4.86 mmol/l. These results suggest that with 90% confidence the growth of any *C. jejuni* strain can be inhibited by the application of 8 mmol/l butyrate treatment at pH 6.0.

5.5.2. Antibiotic-sensitivity of *C. jejuni* strains

Testing the antibiotic sensitivity of the examined *C. jejuni* strains revealed that all strains were found to be sensitive to enrofloxacin. Similarly, growth of most strains was successfully inhibited by ampicillin application, except one (No 420, strain showing outstanding resistance against the antibacterial effect of butyrate at both applied pH as well). In this case no inhibitory zone could be detected, due to ampicillin resistance (**Table 14**).

Table 14. Inhibitory zones gained by agar disk diffusion test with enrofloxacin (5 µg/plate) and ampicillin (10 µg/plate) after 48 h incubation (**Study IV**)

Strain No	Enrofloxacin (mm)	Ampicillin (mm)
1	40.0 ± 0.0	10.0 ± 0.0
3	41.5 ± 0.5	9.0 ± 0.0
93	36.0 ± 0.0	23.0 ± 0.0
420	34.5 ± 0.5	0.0 ± 0.0
615	41.5 ± 0.5	19.5 ± 0.5
1077	15.5 ± 0.5	17.5 ± 0.5
1244	9.0 ± 1.0	38.5 ± 1.5
Reference	41.5 ± 0.5	19.5 ± 0.5

Results are expressed as mean ± standard error of mean (SEM).

6. Discussion

Effect of soluble dietary NSP content on cecal butyrate production was investigated by choosing two cereals (maize and wheat) as bases of diets. High arabinoxylan content of the diet was one of the most probable candidates to enhance intestinal SCFA production and to increase plasma butyrate level in several studies (Ingerslev et al. 2014; Nielsen et al. 2015). In line with this, the WB diet, rich in soluble NSPs and supplemented with the NSP-degrading enzymes xylanase and glucanase (cleaving NSPs to shorter oligosaccharides, also moderating the antinutritive effects of wheat; Selvendran 1984; Hübener et al. 2002), promotes bacterial SCFA production (Wang et al. 2005), but primarily that of butyrate (Molnár et al. 2015) by delivering more oligosaccharide substrates for the cecal microbiome. As an opposite, conventional MB diet represents lower level of soluble NSPs and results in less pronounced butyrate production in the ceca. The higher fiber and NSP content of wheat was suggested by Yang et al. (2020) to stand in the background of the observation that absolute and relative weights of the ceca was higher in broilers fed wheat-based diet, compared to maize-based diet. This phenomenon is presumably based on the direct intestinal effects of butyrate, such as promotion of cell division, growth and consumption as energy source by enterocytes (Roediger 1982; Maclean et al. 1998; Guilloteau et al. 2010). This dietary regime has already been applied in our earlier trials (Kulcsár et al. 2016 and 2017), enabling the comparison of the effects associated with higher (WB) and lower (MB) cecal SCFA production levels. However, it should be taken into account that maize and wheat also greatly differ in some other parameters (such as amino acid and fatty acid profiles), thus the impact of certain further nutrients on the observed diet-associated changes cannot be excluded.

Sodium (n-)butyrate supplementation of diets was applied in order to study the effect of this widely used, exogenous source of butyrate besides that of endogenously produced one.

In contrast to the free butyrate salts, protected types avoid rapid absorption from the proximal part of the gastrointestinal tract, therefore they are able to reach the distal small intestines or even the hindgut (Moquet et al. 2016; Kulcsár et al. 2017). After the release of butyrate from its protected form, it acts directly on the intestinal microflora in the gut, then – after absorption – serves as energy source for the colonocytes or is transported to the liver by the portal circulation. Presumably due to the absorption properties distinct from that of sodium (n)butyrate, protected types often have an effect that is different from what is experienced in case of free butyrate salts.

Beside the effects of soluble NSP content of the diet the influence of the dietary crude protein content was also examined. Diet of LP groups was designed with inclusion of elevated amount of limiting amino acid L-lysine, DL-methionine, L-threonine and L-tryptophan, in order to avoid growth depression caused by inadequate amino acid supply of animals. Therefore,

simultaneously with lowered crude protein content, ratio of free limiting amino acids was higher in these diets, compared to NP groups, resulting in better bioavailability, thus the absorbance of the above mentioned amino acids (Wu 2009).

In **Study I**, the effect of dietary NSP content, feed additive sodium (n)butyrate and crude protein content of the diet was assessed on the general metabolic responsiveness of broilers at the age of 7, 21 and 42 days, to monitor age-dependency.

In **Study II**, the impact of the same dietary factors was investigated on the gene expression and protein abundance of certain members of hepatic insulin and glucagon signaling in 21 days old chickens.

In **Study III**, the modulatory action of feed additive sodium (n)butyrate, its film-coated and fat-embedded protected forms and dietary crude protein content was studied on the weight of carcass traits and chemical composition of the pectoral and femoral muscles at the age of slaughter (d 42).

In **Study IV**, the antimicrobial efficacy of sodium (n)butyrate on different *Campylobacter jejuni* strains was examined at pH 7.4 and 6.0, coupled with antibiotic resistance measurements as background information.

6.1. Study I – Age-dependent general metabolic responsiveness of chickens to nutritional factors

The general responsiveness of animals to dietary treatments showed a remarkable age-dependency. Nutritional factors having impact on any of the response variables could generally alter measured values significantly at the age of 21 days, referring to a metabolism more sensitive to regulatory mechanisms compared to d 7 or 42, presumably due to the intensive growth of the animals in this period of life (Tavares et al. 2015; Murawska 2017).

Although published values are quite diverse in the literature, it is noteworthy to mention that independently from dietary group, measured concentrations and activities of all but one blood plasma parameters were found in the range declared physiological by the majority of relevant articles (Malheiros et al. 2003; Piotrowska et al. 2011; Kowalczuk-Vasilev et al. 2017). The observed significant, but physiological changes are not pernicious, but indicate a metabolic answer of the animals, possibly affecting parameters determining productivity, and as such can not be neglected. The activities of creatine kinase at the age of 42 days were over the physiological value, however, no white stripes or any signs of wooden breast was observed in the pectoral muscle during processing.

Increasing effect of WB diet on total protein levels on d 21 could be associated with a more intensive SCFA production enhanced by wheat (Molnár et al. 2015); however, the exact mechanism of the suggested action is yet unclear. Increased amount of butyrate produced can

be used as substrate for energy production and has the potential to act as an effector in several types of intestinal and liver cells as well (Beauvieux et al. 2001). Although epigenetic or receptor mediated effects of butyrate on liver cells were not investigated in this study, their possible mediating function in hepatic protein synthesis cannot be excluded, and might serve as possible explanation of the slight relapse in albumin production caused by butyrate supplementation, appearing in decreased albumin/TP ratio at the age of 7 days. Low protein diet with limiting amino acid completion has decreased total protein on d 21.

In avian species, uric acid is the major deleterious endproduct of the metabolism of nitrogen containing compounds. Our measurements have shown the age-related gradual decrease of uric acid content of blood plasma in all groups, reflecting the decline of protein degradation rate, in agreement with other observations (Malheiros et al. 2003; Swennen et al. 2005; Hada et al. 2013). Further, WB diet significantly increased the concentration of uric acid at the age of 7 and 21 days, while LP diet had opposite significant effect on d 21 and 42. These findings could be interpreted – in accordance with changes of total protein concentration induced by WB and LP diets – as the consequences of intensive protein metabolism, enhanced by wheat and normal protein level of diet. Lowered crude protein level of diet was reported to decrease blood uric acid concentration by other authors (Rosebrough et al. 1996; Malheiros et al. 2003, Swennen et al. 2005; Namroud et al. 2008; Hada et al. 2013), who explained the phenomenon with diminished protein catabolism under scarce crude protein content of feed. Increasing effect of butyrate supplementation on plasma uric acid concentration on d 21 might be attributed to its epigenetic effect, possibly altering purine degradation of birds.

Stimulation of aspartate aminotransferase activity by WB diet at the age of 21 days coheres well with similar effect of dietary cereal type in the same period of life on uric acid production. Despite that aspartate aminotransferase activity measured from blood plasma means enzymes deliberated from cells, mostly from hepatocytes, no macroscopic signs of liver damage could be observed during the dissection and even this increased enzyme activity was in the physiological range (Chand et al. 2018). Based on these observations it can be assumed that elevated enzyme activity refers to physiologically intensified pathways of protein and amino acid homeostasis and as such, it is not the sign of any harmful effect of the applied dietary strategy. However, the exact mechanism laying in the background of the intensified aspartate aminotransferase activity should be the subject of further investigations.

Plasma creatine kinase activity was elevated by LP, limiting amino acid completed diet on d 21 and 42, and plasma levels increased nearly thirteen times on d 42, compared to d 7 measurement, regardless of diet composition. This age-associated elevation in creatine kinase plasma activities is in line with the findings of Hada et al. (2013) and Malheiros et al. (2003), who explained the phenomenon with increased susceptibility of muscle cell membranes to damage in the phase of intensive growth and similarly, in older animals. We found that chickens

of LP groups were of significantly higher body weight at the age of 21 and 42 days of life compared to NP chickens. This observation can be a potential explanation of the increased creatine kinase activity of animals reared on LP diet supplemented with amino acids, as intensive body weight gain might impair muscle fibers, causing more pronounced muscle cell membrane damage. Plasma creatine kinase activity is of high importance as it was found to be the candidate biomarker to predict the possibility of the formation of white stripes in the breast meat and wooden breast already in the live phase, which is an increasing issue characteristic for fast-growing meat-type broiler lines (Kong et al. 2021). However, despite the supraphysiological values on d 42, no macroscopic signs of muscle damage were observed.

In general, reduced dietary crude protein levels could impair the growth of chickens due to the inadequate amino acid supply (Aletor et al. 2000). However, no decrease was experienced in growth performance when providing amino acids in a well-balanced profile, ensured by essential amino acid supplementation (Aletor et al. 2000). It was also reported by Awad et al. (2014) that dietary crude protein levels could be lowered to a limited extent together with essential amino acid completion to maintain the normal growth and health of broilers. The supplementation of broiler feed with free amino acids is essential in lowering dietary crude protein levels (Pesti 2009), reducing nitrogen excretion (thus the nitrogen load of the environment; Donsbough et al. 2010) but maintaining or even increasing growth and meat production. Congruent with our results, Khan et al. (2011) measured significantly higher body weights for chickens fed low protein, limiting amino acid fortified diet, presumably due to the increased dietary free amino acid to crude protein ratio compared to the diet of control group. Apart from crude protein content of feed, effect of WB diet could be detected, so that animals in WB groups showed higher body weights at the age of 21 and 42 days, compared to their MB counterparts, similarly to the findings of Kulcsár et al. (2016). It is important to highlight that this study was not designed to assess performance parameters, therefore, body weight results must be considered as background data only and interpreted with caution.

Blood glucose level of animals decreased in WB groups on d 21. This change – with no alterations in plasma insulin concentration – might be in connection with the findings of Study II, where WB diet (possibly through more pronounced butyrate exposure of the liver via the portal circulation) proved to trigger increased hepatic IRβ and mTOR levels of 21 days old chickens. The elevation of the protein abundance of these important members of the insulin signaling might refer to the sensitization of the pathway, and therefore, lower level of plasma glucose could be measured even at unchanged concentration of circulating insulin.

Plasma concentration of triglyceride was increased significantly by WB diet at the age of 21 days. This phenomenon might be interpreted as the effect of increased amount of SCFAs, primarily butyrate on liver cells. Butyrate, serving as an alternative source of energy or by its epigenetic effect could promote hepatic lipogenesis and release of triglyceride into the systemic

circulation. The observation could be in association with lowered glucose level, suggesting a relapse in lipid degradation for the energy production (and the possibility of increased lipogenesis for storage, as observed by Griffin et al. 1982) in the cells due to the intensified glucose utilization for the same purpose. On the other hand, increased abundance of IRβ and mTOR proteins caused by WB diet as mentioned above also serve a possible explanation. In the conceivable case that the sensitization of the hepatic insulin signaling pathway results in intensified glucose uptake of the hepatocytes – also reflected by the decline in plasma glucose level –, high amount of acetyl~coA molecule (derived from the glucose degradation) might be involved not only in the energy production, but in non-esterified fatty acid, and as a next step, triglyceride synthesis, which is then released into the systemic circulation.

Elevated levels of GLP-1 and increased concentrations of insulin were experienced on d 21 and 42 compared to d 7 measurements, although to different degree. Presence of high amount of insulin and GLP-1, as an inductor of insulin production may refer to the intensive general metabolic rate of broilers at the age of 21 days, and to a lesser extent, on d 42. Further, the sensitivity of insulin signaling cascade elements decreases with age in birds (Joseph et al. 1996; Deng et al. 2014), that might be another possible explanation of the elevated plasma concentrations of these factors.

Concluding our results, the ability of broiler chickens to respond to nutritional factors has been found strongly age-dependent. Animals have shown particular susceptibility in the phase of intensive growth (at the age of 21 days), indicating that diet composition of animals in the grower phase is critical, consequently, special emphasis should be put on its formulation. We also found that types of basal diet (wheat vs. maize), highly different in their soluble NSP content, are able to affect certain plasma parameters, presumably through the alteration of cecal microbial SCFA, primarily butyrate production. Therefore, dietary cereal type could be considered as a potent effector of the intermediary metabolism, influencing mainly protein and amino acid homeostasis. Further, no adverse effects of slightly reduced dietary crude protein content with limiting amino acid supplementation on growth or health of chickens could be observed, in fact, this reduction may have a beneficial impact from both economic and environmental point of view. However, despite of the significant differences between dietary groups, all but one measured parameters were in the physiological range, thus diet compositions described in the study seem to be applicable safely in poultry nutrition, without disadvantageous impact on the metabolic health and welfare of broilers.

6.2. Study II – Changes in hepatic insulin and glucagon signaling

In this study, the impact of the applied nutritional strategies on selected members of glucagon and insulin homeostasis, namely GCGR, IRβ and mTOR was assessed by measuring gene expression and protein abundance of the listed parameters.

The optimal age of birds for the investigation of the selected parameters was determined according to literature data and based on the results of Study I. Previous studies showed that – independently from the technology and breed applied – the growth rate of broilers is increasing in the middle phase of the life of the animals (Tavares et al. 2015; Murawska,2017). Age-related decrease of the sensitivity of insulin signaling proteins to nutritional factors was also measured in mammals (Gupte et al. 2008) and in birds (Joseph et al. 1996; Deng et al. 2014), while the opposite was observed for glucagon sensitivity in chickens (Joseph et al. 1996). Summarizing all these above described processes and taking into account the 42-day conventional rearing technology, the age of 21 days was considered optimal. On d 21, the metabolism of broilers is intensive with an increasing responsiveness to glucagon, but the sensitivity of insulin signaling elements might be still high enough, therefore, the most relevant information could be expected this age. The results of Study I are also in agreement with this theory, as broilers were found the most responsive to dietary manipulation at the age of 21 days.

An increase of GCGR mRNA expression was observed in WB and LP groups (compared to chickens kept on MB and NP diet, respectively), but these changes could not be detected on protein level. However, exogenous butyrate decreased GCGR content of the hepatocytes without observed alterations of GCGR mRNA production, and the interaction of dietary cereal type and butyrate completion, as well as crude protein content and butyrate supplementation was also detected on protein level. Pairwise comparison showed that GCGR exhibited higher protein levels with no butyrate supplementation in the NP group, and this effect was more pronounced when the animals were fed WB diet. A previous trial showed that butyrate down-regulated GCGR protein abundance of chicken adipocytes in an *in vitro* study (Oscar 1996). Further, the origin (exogenous feed additive or endogenously produced in the intestines) and application form (feed supplementation or bolus) of butyrate could influence the mode of action of this molecule (Mátis et al. 2015; Kulcsár et al. 2016). Although epigenetic or receptor mediated effects of butyrate were not investigated in this study, one possible explanation for the apparent inconsistency of mRNA and protein levels might be the butyrate-evoked partial inhibition of the post-transcriptional processing of GCGR mRNA.

Concerning the expression data of IRβ and mTOR as prominent members of the insulin signaling pathway, IRβ mRNA concentration was significantly affected by the interaction of all dietary factors, while mTOR mRNA level increased in WB and in LP groups. However, an increase in protein level of both elements was observed in WB groups, while butyrate supplementation of the diet decreased hepatic IRβ protein abundance, and the interaction of dietary cereal and crude protein content of the diet, as well as crude protein content and butyrate supplementation was also detected. Interestingly, WB diet could exert its IRβ protein abundance inducing effect more when combined with NP, and butyrate also had a more

pronounced decreasing effect in the NP groups, suggesting that independent of the origin, butyrate was more potent in affecting hepatic IRβ protein abundance in the NP groups. Despite the higher crude protein content of NP diet, it can be of some aspects considered as a diet with less favorable composition due to the lack of easily available limiting amino acids, and it is conceivable that similarly to other observations, butyrate has a more notable influence under suboptimal conditions (Leeson et al. 2005).

Although the structure and function of insulin receptors in birds are similar to those of mammals (Dupont et al. 2009), the physiological protein levels of both receptor subunits and IRS-1, as well as the inducibility of the insulin cascade mechanism are lower in chicken than in mammalian species (Dupont et al. 2012). Because of this moderate responsiveness to insulin, the observed increase of IRβ and mTOR protein abundance in WB groups can be of outstanding importance in the activation of downstream elements of the signaling pathway, suggesting the opportunity of finding new ways of growth promotion in broiler industry via the amelioration of insulin sensitivity. Notwithstanding that the present study was not designed to assess performance data, it should be noted as background information that body weight of chickens at the age of 21 days was increased by applying WB and LP (limiting amino acid fortified) diets, as described at **Study I**. These results are in accordance with those of previous studies (Kulcsár et al. 2017; Mátis et al. 2019), and might be partly related to the detected amelioration of the hepatic IRβ and mTOR protein abundance in WB groups.

It is highlighted by the present results that the type of cereal could exert modulatory action on IRβ and mTOR expression on both mRNA and protein level; however, this effect highly depended on the crude protein content of the applied diet in the case of IRβ. The increased amount of IRβ and mTOR protein in the liver might be a result of the microbial butyrate synthesis enhanced by the soluble NSP content of WB diet in the ceca. In this respect, our results are in line with the observation of Kulcsár et al. (2016), who also found elevated hepatic IRβ and mTOR protein levels in the animals of WB group at the age of 42 days. Endogenously produced SCFAs, primarily butyrate was shown to increase insulin sensitivity and glycemic control in several *in vivo* studies with mammals (Boll et al. 2016; Miyamoto et al. 2018). Further, Ramiah et al. (2019) found increased mTOR mRNA level in the liver of broiler chickens, mentioning high NSP level of the feed as a possible explanation. In **Study I.**, we measured lowered blood glucose level of the animals in WB group at the age of 21 days. However, we did not detect any change in the plasma insulin concentration of WB group compared to MB group, which is in correspondence with the results of Józefiak et al. (2010), who also reported increased insulin sensitivity of the liver of broiler chickens fed wheat-soybean-based, enzyme supplemented diet without any effect on plasma insulin level. Further, sodium butyrate proved to promote the phosphorylation of Akt, the direct activator of mTOR in IPEC-J2 cell line (Yan and Ajuwon 2017) and in the liver of mice (Mollica et al. 2017), as well as to enhance Akt

activation and assembly of rictor (rapamycin insensitive companion of mTOR) and mTOR complex in insulin-resistant HepG2 human cells (Endo et al. 2013; Mollica et al. 2017). Although we detected a diminishing effect of exogenous sodium butyrate on IRβ on protein level and no effect was observed on mTOR on mRNA or protein level, future investigation of the phosphorylation state of these two insulin signaling elements could reveal whether butyrate supplementation of the diets altered their activation. Further, the observed interactions highlight that feed additives might exert distinct actions under various dietary conditions; therefore, the complex interplay of different dietary factors should be addressed to improve animal health and productivity by novel nutritional strategies.

Similarly to the observations of GCGR, mTOR mRNA concentrations were increased in LP groups, but this effect was not realized on mTOR protein production level. In line with our results, it was reported that restricted dietary protein supply might affect the expression of certain genes without influencing protein quantities in swine (Jia et al. 2016; Shifeng et al. 2018). Additionally, the capacity of low-protein diet to alter expression of metabolism-related genes was also detected in studies conducted on broilers (Adams and Davis 2001; Kita et al. 2002).

Concluding our results, dietary cereal type remarkably influenced the hepatic endocrine metabolic regulation of broilers in the phase of intensive growth. Wheat-based diet successfully increased the quantity of the investigated members of the insulin signaling pathway on protein level, with an even more pronounced effect in the NP groups in the case of IRβ. This finding is of outstanding importance, indicating increased hepatic insulin sensitivity, which might be in certain cases beneficial due to the growth promoting effect of insulin. Further, butyrate as a feed additive proved to alleviate hepatic GCGR and IRβ protein abundance, while WB diet and lowered crude protein level were able to increase GCGR gene expression on the level of transcription only, highlighting that the origin of butyrate might influence its mode of action. The obtained results might contribute to the better understanding of glycemic control of birds and to the opportunity of improving glucose homeostasis, enhancing production parameters and welfare of broiler chickens. It can be concluded that dietary factors, particularly the cereal type play pivotal role in the modulation of the endocrine regulation of the liver in chickens, serving as a key link between nutrition and metabolic health, but the complex interaction of different dietary factors cannot be neglected. Hence, these factors should be considered as useful tools to improve animal health and productivity, which can be applied to promote sustainable poultry production.

6.3. Study III – Nutritional effects on the weights of organs and carcass characteristics

Butyrate as a feed additive, both in its unprotected or protected form, had a stimulatory action on the growth of broilers and had a remarkable influence on the chemical composition of thighs. Our results showed that live weight and carcass weight were significantly increased by all protected butyrate forms, and relative breast meat yield was elevated in chickens fed unprotected and protected butyrate as well, compared to controls without butyrate addition.

The stimulatory action of butyrate on broiler meat production has already been shown in several studies, measuring increased carcass yield of butyrate-supplemented animals (Leeson et al. 2005; Hu and Guo 2007; Panda et al. 2009). In our study, all the applied protected butyrate products were able to increase carcass weight, while unprotected butyrate could not provoke the significant alteration of this parameter. However, relative breast meat yield was elevated by both unprotected and protected butyrate supplementation, indicating higher mass and a higher proportion of pectoral muscle among meat types. The absolute breast meat mass, as a sum of the increased carcass weight and relative breast meat yield, was also elevated (with approximately 34 %) by all types of protected butyrate application (breast meat mass in butyrate free controls, NP Ctr group: 491:4 ± 33:0 g; in NP S90 group: 655:3 ± 29:9; in NP SC40 group: 657:0 ± 29:1 g; in NP SC30 group: 663:6 ± 19:3 g).

The growth-promoting action of butyrate can be related to its several beneficial biological effects. In the intestines, butyrate enhances the development of the intestinal mucosa (Antongiovanni et al. 2007; Adil et al. 2010), increases the barrier function of the gut wall (Wang et al. 2012) and maintains intestinal microflora by selectively inhibiting the growth of certain pathogenic bacteria (Hu and Guo 2007). The greater intestinal absorptive capacity and more balanced microflora may contribute to the increased growth and altered carcass characteristics. Further, the absorbed butyrate can act as an epigenetically active molecule in several tissues and may also elicit some receptor-mediated effects (Mátis et al. 2013b). For instance, the butyrate-evoked modulation of insulin homeostasis can also be related to stimulated muscle development as insulin receptor β protein abundance was selectively upregulated in skeletal muscle after oral butyrate application in bolus, presumably leading to increased insulin sensitivity (Mátis et al. 2015).

The lipid content of the femoral muscle was increased and the protein content has changed inversely by all types of butyrate applied, but no changes were observed in the chemical composition of the breast meat. The observed alterations in thighs may improve meat quality, moreover the increased muscular lipogenesis was not coupled with abdominal fat deposition, undesirable for poultry industry. A similar action of orally applied butyrate was also described in feedlot cattle, where the lipid content, thus the marbling of the meat significantly

increased as the consequence of calcium butyrate supplementation of the feed (Moreira et al. 2016).

No relevant differences were found between the efficacy of different protected butyrate types, nevertheless, the tested protected products seemed to be more effective in certain cases than unprotected free butyrate salt, such as in stimulating total carcass weight (where unprotected butyrate failed to exert such effect) or in increasing breast meat yield to a higher extent than unprotected butyrate. The explanation of this phenomenon is that dietary completion of protected butyrate ensures butyrate release in more distal sections of the intestines, while the rapid absorption of unprotected butyrate starts already in the crop and is completed in the duodenum (Kulcsár et al. 2017). The special kinetic properties of protected butyrate products should deliver better butyrate exposure for the intestinal microflora, and the prolonged absorption may also have distinct effects on various extraintestinal tissues compared to the action of unprotected butyrate (Kulcsár et al. 2016 and 2017). Similarly, protected butyrate was capable to increase body weight of the animals at the age of 21 and 42 days, while unprotected butyrate not (**Study I**).

According to our results, the low-protein, amino-acid supplemented diet increased the live body weight (on d 21 and 42), further, carcass weight and relative breast meat yield of broilers on d 42. As detailed at **Study I**, reduced dietary crude protein levels with improper amino acid composition could impair the growth of chickens, but this limitation can be overcome by essential amino acid supplementation (Aletor et al. 2000) and therefore, dietary crude protein content can be diminished to some extent with concomitant limiting amino acid completion without any harmful effect on the growth and health of chickens (Awad et al. 2014). Khan et al. (2011) also measured significantly greater body weights in broilers fed LP, limiting amino acid fortified diet. The elevation of the relative breast meat yield in broilers kept on an LP, limiting amino acid fortified diet indicated that not only the absolute mass but also the proportion of breast meat as the most valuable part of the chicken carcass could be increased by this dietary strategy. This hypothesis is supported by several studies (Tesseraud et al. 1996 and 2001; Urdaneta-Rincon and Leeson 2004), describing that dietary Lys supplementation modifies the balance between protein synthesis and degradation, enhancing muscle growth in chicken skeletal muscles, especially in the pectoralis major muscle.

In our study, the weight of liver was significantly increased by the LP diet. In contrast, Awad et al. (2014) found no alteration in liver mass when low-protein and amino-acid-fortified diets were given to broilers; however, they investigated chickens in the grower phase and applied slightly lowered dietary lysine levels compared to this examination. As observed in the present **Study III**, the protein content of the femoral muscle was significantly decreased, while its lipid content was increased by the LP diet when compared to NP animals. However, relative abdominal fat mass was not affected by dietary crude protein levels. Presumably due to the

lower dietary protein and higher carbohydrate content of the feed (to gain isoenergetic diets), the lipogenesis could be stimulated in LP groups. However, the subsequently increased triglyceride disposition was realized in the femoral muscle only, improving meat quality and did not result in greater abdominal fat reserves. In another study, various isoenergetic LP diets (from 23 % to 18% crude protein) increased the lipid content of the whole carcass independently of the provided amino acid supplementation in 21-day-old broilers (Bregendahl et al. 2002). Similarly, abdominal fat mass and the amount of extractable carcass fat were increased by lower dietary crude protein levels (from 25 % to 18 %) but were reduced by arginine or lysine completion in the starter phase (Hurwitz et al. 1998). The underlying mechanism is that when an essential amino acid, and especially Lys is not ensured at a sufficient level in the diet, protein synthesis is limited and the surplus amino acids – that are not incorporated into proteins – are catabolized and used in the lipid pathway, resulting fat deposition (Grisoni et al. 1991; Leclercq 1998; Nasr and Kheiri 2012).

The effects of unprotected sodium butyrate were compared in chickens kept on an LP or NP diet to gain some preliminary data about the possible interaction of various dietary factors and their impact on carcass characteristics. According to our results butyrate altered the chemical composition of the femoral muscle similarly both in LP and NP groups. However, the stimulatory action of unprotected butyrate on breast meat yield was lacking in the case of the lowered crude protein supply with essential amino acid supplementation. The partly different action of butyrate in in this aspect cannot be explained based on these data. Nonetheless, it can be hypothesized that different amino acid content of the diet may alter the composition of the intestinal microflora, possibly interfering with the utilization of exogenously applied butyrate and the endogenous microbial butyrate production. Further, orally applied butyrate may also influence the pH of the ingesta, possibly acting on protein digestion and utilization as well. Based on our results, it must be highlighted that feed additives such as butyrate can elicit different effects under various dietary conditions; thus, combining nutrition strategies in order to optimize animal production should be considered carefully. Therefore, based on these initial results about the combination of sodium butyrate as a pure substance and altered dietary crude protein supply, future studies are needed in terms of the possible interactions of different butyrate-containing products and dietary crude protein levels or cereal types.

In contrast to the thighs, no diet associated changes (either in the case of butyrate addition or dietary crude protein levels) could be detected in the chemical composition of the pectoral muscle. This finding might be connected to different muscle fiber composition and metabolic properties of these two muscles and suggests a great stability of breast meat composition.

Based on our results, it can be concluded that the development and production of breast meat can be effectively stimulated by decreased dietary crude protein content with limiting

amino acid fortification, as well as by unprotected and protected butyrate supplementation, but its chemical composition remains unchanged. As an opposite, the same factors are suitable for the amelioration of femoral muscle composition, but exert no significant effect on its weight, and only unprotected butyrate failed to significantly elevate carcass yield. Therefore, the above mentioned dietary strategies seem to be nutritional tools to selectively change carcass characteristics of broilers.

6.4. Study IV – The in vitro antibacterial efficacy of butyrate and antibiotic sensitivity of *C. jejuni* strains

Butyrate showed a remarkable inhibiting effect on most of the investigated *C. jejuni* strains in the established *in vitro* model, showing strong pH-dependency: MIC and MBC values of butyrate decreased intensively in slightly acidic milieu (at pH 6.0) compared to those measured at pH 7.4. This is in agreement with the results of van Deun et al. (2008a), who found butyrate being bactericidal at more acidic pH already in lower concentrations. The key role of the pH can be explained by the dissociation properties of butyrate: at lower pH, more molecules remain undissociated in the form of butyric acid, penetrating through the bacterial membranes easier by simple diffusion than dissociated butyrate anions. These results highlight the importance of pH in the cecal ingesta *in vivo* as well, which should also be decreased in order to reach optimal efficacy. Amongst others, cecal pH can be influenced by special dietary conditions, such as by the application of various NSPs or feeding NSP-rich diet (such as rye, barley or wheat) and carbohydrase enzyme completion of the feedstuff (Angkanaporn et al. 1994; van Beilen and Li 2002).

According to our *in vitro* results, butyrate could be an effective tool in the amelioration of *Campylobacter* colonization in broilers, as butyrate concentration that is multiple of its MIC value – measured *in vitro* at pH 6.0 as 5.0 mml/l– was reached in the ceca of broilers at the age of 42 days when fed WB diet (Kulcsár et al. 2017). Further, the butyrate sensitivity of the tested reference strain was comparable with that of most field isolates, therefore, despite minor differences, the obtained MIC and MBC values in general might be a base for the estimation of approximate butyrate sensitivity of other Central European *C. jejuni* strains as well.

However, direct extrapolation of the obtained *in vitro* results to *in vivo* application is restricted due to numerous factors, which may modulate the antibacterial efficacy of butyrate. First of all, it is essential to reach the required butyrate concentration in the cecal chyme, which is affected by the intestinal absorption as well. Due to the extensive removal of organic acids from the gut, orally added L-lactate proved to be unable to reach the cecum and therefore its supplementation in drinking water failed to reduce cecal *Campylobacter* count (Byrd et al. 2001; Heres et al. 2003 and 2004; van Deun et al. 2008a). Van der Wielen et al. (2000) found that

the number of enterococci and *Enterobacteriaceae* species were in negative correlation with the butyrate concentration of the ceca of broiler chickens. Unlike free salts, protected forms of butyrate and other organic acids bypass the absorption from the proximal section, reaching the distal part of the gastrointestinal tract. Such butyrate-coated micro-beads were already successfully applied in *Salmonella* eradication programs in chicken (van Immerseel et al. 2006), and our previous studies also show that protected butyrate is capable to elevate the ileal butyrate content significantly (Kulcsár et al. 2017). A similar application would be possible to use protected butyrate in *Campylobacter* controlling as well, probable in combination with increased uptake of some additional feed components enhancing cecal bacterial fermentation (e.g. SCFA-precursors, enzyme supplementation). Bypass carbohydrates, such as NSPs or resistant starch, being undegradable in the small intestines, can serve as precursors for microbial SCFA synthesis. Increased NSP or resistant starch content of the diet can therefore enhance the endogen microbial butyrate production, which may result in elevated cecal butyrate concentration and decreased pH, intensifying the antibacterial property of butyrate (Guilloteau et al. 2010).

It is known that the mucous layer reduced the potency of butyrate by increasing the MIC values *in vitro* (van Deun et al. 2008a). As a consequence, oral application of protected butyrate could not effectively ameliorate the cecal colonization of the investigated *C. jejuni* strain in an *in vivo* trial with broiler chicks (van Deun et al. 2008a). However, intestinal pH and butyrate concentration were not studied in this trial, which factors would be of predominant importance by influencing the efficacy of the treatment.

Van Deun et al. (2008c) showed that butyrate protected the intestinal cell line Caco-2 against the invasion and translocation of a *C. jejuni* strain, but failed to protect it against the *C. jejuni*-specific negative effects on cellular integrity and barrier function of the gut. Reducing *Campylobacter* colonization is adequate to decrease the contamination of meat, however, integrity of the intestinal barrier is also an important issue to improve the health of the animals.

Despite that only 5.5% of *C. jejuni* and *C. coli* strains showed ampicillin resistance in a study conducted in the early 1990's (Cabrita et al. 1992), this phenomenon became worryingly common among *C. jejuni* field isolates of poultry origin, which can be combined with multi-drug resistance in several cases (Kim et al. 2010; Kittl et al. 2011). Despite the fact that most *C. jejuni* strains of this study proved sensitive to the tested antibiotics, due to the demand on reducing antibiotics administration in food producing livestock, application of traditional antimicrobials are undesirable in *Campylobacter* eradication programs (van Immerseel et al. 2006). Thus, SCFAs in general and first of all their most potent representative butyrate, might serve as a suitable alternative of traditional antibiotics.

Based on our results and the cited literature data, orally administered butyrate and dietary factors such as high NSP content (Choct and Annison 1992), pro- and prebiotics mixed in the

feed (Chichlowski et al. 2007), as well as certain exogenous carbohydrase enzymes (Fernandez et al. 2000) – modifying cecal fermentation and thus pH – might be useful tools to reduce enteral *C. jejuni* colonization. It can be concluded that butyrate in appropriate concentration and at lower pH values acts effectively against most *C. jejuni* strains *in vitro*. However, many enteral factors might have impact on the practical use of this substance, therefore, further studies are required in terms of the *in vivo* application of butyrate, with special emphasis on the proper form and dose, ensuring a cecal butyrate concentration optimal to fully exert its antibacterial efficacy.

7. New scientific results

Ad 1,

Dietary cereal type and crude protein content significantly influenced the major metabolic blood parameters of broiler chickens, being the most pronounced on d 21. However, all diet-associated metabolic changes were found within the physiological range.

Ad 2,

In the phase of intensive growth (21 days of age), wheat-based diet – compared to maize-based diet – showed to increase the protein abundance of IRβ and mTOR insulin signaling proteins in the liver of broilers, thus potentially enhance the hepatic insulin sensitivity. Unprotected sodium n-butyrate as feed additive could decrease both hepatic GCGR and IRβ protein expressions.

Ad 3,

The production of breast meat of broiler chickens could be efficiently stimulated by 15% lowered dietary crude protein content of the diet supplemented with limiting amino acids and by the application of either protected or unprotected (n-)butyrate, but its chemical composition remained unchanged. In contrast, the same diets altered the femoral muscle composition without affecting relative thigh yield significantly, but proved to increase carcass weight of broilers.

Ad 4,

Sodium (n-)butyrate exerted antibacterial effects against most *C. jejuni* strains in vitro at pH 6.0 in 5 mmol/ml (MIC) and 5 to 7.5 mmol/l (MBC) concentrations which are reachable by adequate diet formulation in the intestines of live broilers.

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9. Own scientific publications

9.1. Publications related to the topic of the present dissertation

9.1.1. Full text papers in peer-reviewed journals

Petrilla, J.; Mátis, G.; Mackei, M.; Kulcsár, A.; Sebők, Cs.; Papp, M.; Gálfi, P.; Fébel, H.; Huber, K.; Neogrády, Zs.: **Modulation of Hepatic Insulin and Glucagon Signaling by Nutritional Factors in Broiler Chicken.** *Veterinary Sciences,* 9. 103, 2022.

Petrilla, J.; Mátis, G.; Molnár, A.; Jerzsele, Á.; Pál, L.; Gálfi, P.; Neogrády, Zs.; Dublecz, K.: **A butirát antibakteriális hatékonyságának *in vitro* vizsgálata különféle *Campylobacter jejuni* törzseken (Investigation of *in vitro* antibacterial efficacy of the short chain fatty acid butyrate on various *Campylobacter jejuni* strains).** *Magyar Állatorvosok Lapja,* 143. 57–64, 2021.

Mátis, G.; Petrilla, J.; Kulcsár, A.; Van den Bighelaar, H.; Boomsma, B.; Neogrády, Zs.; Fébel, H.: **Effects of dietary butyrate supplementation and crude protein level on carcass traits and meat composition of broiler chickens.** *Archives Animal Breeding,* 62. 527–536, 2019.

Petrilla, J.; Mátis, G.; Kulcsár, A.; Talapka, P.; Bíró, E.; Mackei, M.; Fébel, H.; Neogrády, Zs.: **Effect of dietary cereal type, crude protein and butyrate supplementation on metabolic parameters of broilers.** *Acta Veterinaria Hungarica*, 66. 408–452, 2018.

9.1.2. Oral and poster presentations on international conferences

Petrilla, J.; Mátis,G.; Kulcsár, A.; Talapka,P.; Bíró, E.; Mackei, M.; Fébel, H.; Neogrády, Zs.: **The effect of dietary cereal type, crude protein content and butyrate application on selected markers of metabolism in broiler chickens.** GfE (Society of Nutrition Physiology) Conference**,** Göttingen, Germany, 2017.

9.1.3. Oral presentations on Hungarian national conferences

Kulcsár, A.; Sebők, Cs.; Mátis, G.; Talapka, P.; Hatala, P.; Petrilla, J.; Fébel, H.; Neogrády, Zs.: **Az inzulin és a glukagon jelpálya különböző takarmányozási faktorok segítségével történő szabályozása brojlercsirkében.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2018.

Kulcsárné Petrilla, J.; Mátis, G.; Kulcsár, A.; Talapka, P.; Bíró, E.; Mackei, M.; Fébel, H.; Neogrády. Zs.: **Metabolikus paraméterek változásai a takarmánygabona típusa, a**

takarmány nyersfehérje-tartalma és butirátkiegészítés hatására csirkében. MTA Akadémiai Beszámolók, Budapest, Hungary, 2017.

Mátis, G.; Kulcsár, A.; Kulcsárné Petrilla, J.; Talapka, P.; Bardóczy, M.; Mackei, M.; Neogrády, Zs.; Fébel, H.: **Egyes takarmányozási tényezők brojlercsirkék testösszetételére gyakorolt hatásának vizsgálata.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2017.

Kulcsár, A.; Molnár, A.; Mátis, G.; Petrilla, J.; Pál, L.; Jerzsele, Á.; Neogrády, Zs.; Dublecz, K.: **A különböző koncentrációban adott butirát gatló hatásának *in vitro* vizsgálata Campylobacter jejuni törzsekre.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

9.2. Publications not related to the topic of the present dissertation

9.2.1. Full text papers in peer-reviewed journals

Mátis, G.; Kulcsár, A.; Mackei, M.; Petrilla, J.; Neogrády, Zs.: **Comparative study on the modulation of insulin and incretin homeostasis by butyrate in chicken and rabbit.** *PLoS ONE*, 13. e0205512, 2018.

Kulcsár, A.; Mátis, G.; Molnár, A.; Petrilla, J.; Wágner, L.; Fébel, H.; Husvéth, F.; Dublecz, K.; Neogrády, Zs.: **Nutritional modulation of intestinal drug-metabolizing cytochrome P450 by butyrate of different origin in chicken.** *Research in Veterinary Science,* 113. 25–32, 2017.

Mátis, G.; Kulcsár, A.; Petrilla, J.; Talapka, P.; Neogrády, Zs.: **Porcine hepatocyte-Kupffer cell co-culture as an in vitro model for testing the efficacy of anti-inflammatory substances.** *Journal of Animal Physiology and Animal Nutrition,* 101. 201–207, 2017.

Kulcsár, A.; Mátis, G.; Kulcsárné Petrilla, J., Neogrády, Zs.: **A bélnyálkahártya szerepe a xenobiotikumok metabolizmusában, különös tekintettel a citokróm P450 enzimrendszerre. Irodalmi áttekintés (The role of intestinal mucosa in the metabolism of xenobiotics with particular regard to the cytochrome P450 enzyme system. Literature review).** *Magyar Állatorvosok Lapja,* 138. 243–250, 2016.

Kulcsár, A.; Mátis, G.; Molnár, A.; Petrilla, J.; Husvéth, F.; Huber, K.; Dublecz, K.; Neogrády, Zs.: **Effects of butyrate on the insulin homeostasis of chickens kept on maize- or wheat-based diets.** *Acta Veterinaria Hungarica,* 64. 482–496, 2016.

Mátis, G.; Kulcsár, A.; Petrilla, J.; Hermándy-Berencz, K.; Neogrády, Zs.: **Feed-drug interaction of orally applied butyrate and phenobarbital on hepatic cytochrome**

P450 activity in chickens. *Journal of Animal Physiology and Animal Nutrition,* 100. 637–642, 2016.

Mátis, G.; Hatala. P.; Kulcsár, A.; Kulcsárné Petrilla, J.; Neogrády, Zs.: **A Kupffer-sejtek szerepe a máj gyulladásos és metabolikus folyamatainak szabályozásában. Irodalmi áttekintés (Role of Kupffer-cells in the regulation of hepatic inflammatory and metabolic processes. Literature review).** *Magyar Állatorvosok Lapja*, 137. 569–575, 2015.

Farkas, O.; Mátis, G.; Pászti-Gere, E.; Palócz, O.; Kulcsár, A.; Petrilla, J.; Csikó, Gy.; Neogrády, Zs.; Gálfi, P.: **Effects of Lactobacillus plantarum 2142 and sodium n-butyrate in LPS-triggered inflammation: comparison of IPEC-J2 and primary hepatocyte mono-cultures with a porcine enterohepatic co-culture system.** *Journal of Animal Science,* 92. 3835–3845, 2014.

Mátis, G.; Lengyel, P.; Kulcsár, A.; Kulcsárné Petrilla, J.; Neogrády, Zs.: **A szénhidrát-anyagcsere és az inzulin-homeosztázis sajátosságai csirkében. Irodalmi összefoglaló (Special characteristics of carbohydrate metabolism and insulin homeostasis in chicken. Literature review).** *Magyar Állatorvosok Lapja*, 136. 342–349, 2014.

Mátis, G.; Csikó, Gy.; Jemnitz, K.; Veres, Zs.; Fébel, H.; Kulcsár, A.; Petrilla, J.; Neogrády, Zs.. **A takarmányba kevert butirát citokróm P450 enzimekre gyakorolt hatásának vizsgálata patkány májban (Investigation of the effect of butyrate supplementation of the diet on hepatic cytochrome P450 enzymes in rats).** *Magyar Állatorvosok Lapja*, 135. 109–116, 2013.

9.2.2. Oral and poster presentations on international conferences

Mackei, M.; Kulcsárné Petrilla, J.; Orbán, K.; Neogrády, Zs.; Mátis, G.; Fébel, H.: **Modulation of plasma incretin and insulin levels by oral butyrate application in chicken and rabbit.** 23rd Congress of the European Society of Veterinary and Comparative Nutrition, Turin, Italy, 2019.

Kulcsár, A.; Mátis, G.; Petrilla, J.; Talapka, P.; Fébel, H.; Neogrády, Zs.; Huber, K.: **Influencing insulin homeostasis of broiler chicken by maize- or wheat-based diets.** XXth World Veterinary Poultry Association Congress, Edinburgh, Scottland, 2017.

Kulcsár, A.; Mátis, G.; Petrilla, J.; Talapka, P.; Neogrády, Zs.; Huber, K.: **Effect of maize- or wheat-based diets on the abundance of selected proteins involved in insulin signaling of broiler chicken.** GfE (Society of Nutrition Physiology) Conference, Göttingen, Germany, 2017.

Mátis, G.; Kulcsár, A.; Petrilla, J.; Talapka, P.; Bardóczy, M.; Mackei, M.; Neogrády, Zs.; Fébel, J.: **Investigations on the effects of certain nutritional factors on carcass composition of broiler chickens.** GfE (Society of Nutrition Physiology) Conference, Göttingen, Germany, 2017.

Kulcsár, A.; Mátis, G.; Petrilla, J.; Mackei, M.; Neogrády, Zs.: **Comparative examinations on the nutritional modulation of incretin and insulin secretion in chicken and rabbit.** DVG Conference, Berlin, Germany, 2016.

Mátis, G.; Kulcsár, A.; Molnár, A.; Petrilla, J.; Wágner, L.; Dublecz, K.; Neogrády, Zs.: **Butyrate of different origin affects intestinal drug-metabolizing cytochrome P450 enzymes in chicken.** XXV World's Poultry Science Congress, Peking, China, 2016.

Mátis, G.; Kulcsár, A.; Petrilla, J.; Orbán, K.; Neogrády, Zs.: **Porcine hepatocyte – Kupffer-cell co-cultures as in vitro models for testing the efficacy of anti-inflammatory molecules.** DVG Conference, Physiology and Biochemistry, Berlin, Germany, 2016.

Mátis, G.; Kulcsár, A.; Petrilla, J.; Neogrády, Zs.: **Nutritional modulation of insulin and incretin homeostasis by butyrate in broiler chickens.** GfE (Society of Nutrition Physiology) Conference, Göttingen, Germany, 2015.

Mátis, G.; Kulcsár, A.; Petrilla, J.; Neogrády, Zs.: **Establishment of a porcine hepatocyte – Kupffer cell co-culture as a novel inflammatory model in veterinary research.** XVIIIth International Symposium on Cells of the Hepatic Sinusoid, Asilomar, United States of America, 2015.

Hermándy-Berencz, K.; Mátis, G.; Petrilla, J.; Kulcsár, A.; Huber, K.; Neogrády, Zs.: **How can butyrate modify the induced cytochrome P450 enzyme activity in the liver of chicken?** GfE (Society of Nutrition Physiology) Conference, Göttingen, Germany, 2014.

Mátis, G.; Kulcsár, A.; Petrilla, J.; Kenéz, Á.; Csikó, Gy.; Neogrády, Zs.; Huber, K.: **Epigenetic consequences of oral butyrate application in chicken.** GfE (Society of Nutrition Physiology) Conference, Göttingen, Germany, 2013.

Mátis, G.; Pászti-Gere, E.; Farkas, O.; Kulcsár, A.; Palócz, O.; Petrilla, J.; Csikó, Gy.; Neogrády, Zs.; Gálfi, P.: **Effects of LPS challenge and the role of probiotics in IPEC-J2 cell monoculture and a novel porcine enterohepatic co-culture system.** International Scientific Conference on Probiotics and Prebiotics, Košice, Slovakia, 2013.

9.2.3. Oral presentations on Hungarian national conferences

Kulcsár, A.; Mátis, G.; Kulcsárné Petrilla, J.; Talapka, P.; Neogrády, Zs.; Huber, K.: **A kukorica és búza alapú takarmány hatása az inzulin-homeosztázisra brojlercsirkében.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2017.

Mátis, G.; Kulcsár, A.; Kulcsárné Petrilla, J.; Talapka, P.; Hatala, P.; Mackei, M.; Neogrády, Zs.: **A hőstressz sejtszintű hatásainak vizsgálata csirke májsejt – Kupffer-sejt ko-kultúrán.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2017.

Kulcsár, A.; Mátis, G.; Petrilla, J.; Mackei, M.; Neogrády, Zs.: **Az inkretin és inzulin szekréció eltérő alakulása csirkében és nyúlban: összehasonlító vizsgálatok.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2016.

Mátis, G.; Kulcsár, A.; Kulcsárné Petrilla, J.; Orbán, K.; Neogrády, Zs.: **Terpinen-4-ol és nátrium n-butirát gyulladáscsökkentő hatásának vizsgálata májsejt – Kupffer-sejt ko-kultúrákon.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2016.

Kulcsár, A.; Mátis, G.; Molnár, A.; Kulcsárné Petrilla, J.; Farkas, O.; Wágner, L.; Fébel, H.; Neogrády, Zs.: **Enterális CYP enzimek aktivitásának változása a (n-)butirát különböző alkalmazási formáinak hatására csirkében.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2015.

Mátis, G.; Kulcsár, A.; Kulcsárné Petrilla, J.; Hatala, P.; Kővágó, Cs.; Neogrády, Zs.: **Bakteriális lipopoliszacharidok által kiváltott gyulladás vizsgálata sertés hepatocyta – Kupffer-sejt ko-kultúra modellen.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2015.

Mátis, G.; Kulcsár, A.; Kulcsárné Petrilla, J.; Lengyel, P.; Mackei, M.; Neogrády, Zs.: **Az inzulin- és az inkretin-homeosztázis befolyásolása nutritív faktorokkal csirkében.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2015.

Mátis, G.; Kulcsár, A.; Kulcsárné Petrilla, J.; Hatala, P.; Kővágó, Cs.; Csikó, Gy,; **Neogrády, Zs.: A Kupffer-sejtek arányának meghatározása sertés primer májsejttenyészeten.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2014.

Mátis, G.; Kulcsárné Petrilla, J.; Kulcsár, A.; Hermándy-Berencz, K.; Csikó, Gy.; Neogrády, Zs.: **Befolyásolja-e a butirát az indukált citokróm P450 aktivitást a csirke májában?** MTA Akadémiai Beszámolók, Budapest, Hungary, 2014.

Palócz, O.; Farkas, O.; Csikó, Gy.; Pásztiné Gere, E.; Mátis, G.; Kulcsár, A.; Petrilla, J.; Neogrády, Zs.; Gálfi, P.: **Probiotikumok hatása LPS kiváltotta gyulladás mellett in**

vitro bélhám – májsejt kokultúra modellen. MTA Akadémiai Beszámolók, Budapest, Hungary, 2014.

Farkas, O.; Palócz, O.; Csikó, Gy.; Pásztiné Gere, E.; Mátis, G.; Kulcsár, A.; Petrilla, J.; Neogrády, Zs.; Gálfi, P.: **Oxidatív stress és gyulladás hatásának vizsgálata *in vitro* bélhám- és májsejt ko-kultúra modellen.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

Mátis, G.; Csikó, Gy.; Kulcsár, A.; Petrilla, J.; Farkas, O.; Palócz, O.; Neogrády, Zs.; Gálfi, P.: **Bakteriális lipopoliszacharidok által kiváltott oxidatív stressz hatása sertés primer májsejttenyészetre.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

Mátis, G.; Csikó, Gy.; Kulcsár, A.; Petrilla, J.; Pleva, D.; Neogrády, Zs.; Gálfi, P.: **Máj citokróm P450 enzimek aktivitásanak vizsgálata bolusban adott butirátkezelést követően brojlercsirkében.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

9.2.4. Supervision of DVM theses

Bíró Enikő: **Metabolikus paraméterek változásai a takarmánygabona típusa, a takarmány nyersfehérje-tartalma és butirátkiegészítés hatására csirkében.** TDK dolgozat, 2016. Supervisors: Kulcsárné dr. Petrilla Janka, Neogrády Zsuzsanna

10. Supplementary materials

Table S1. Linear model coefficient estimates and their standard errors fitted to the gene expression and protein abundance data for each analyzed signaling element.

Glucagon receptor				
	Gene expression		**Protein abundance**	
	Estimate	**SE**	**Estimate**	**SE**
Intercept	0.1543	0.0422	1.3519	0.2712
Cereal-WB	0.0195	0.0596	1.0107	0.3940
Protein-LP	0.0773	0.0581	-0.3828	0.3835
Butyrate-But	0.0116	0.0596	-0.5123	0.3835
Cereal-WB:Protein-LP	0.1269	0.0832	-0.4942	0.5498
Cereal-WB:Butyrate-But	0.0549	0.0832	-0.8763	0.5572
Protein-LP:Butyrate-But	-0.0125	0.0822	0.9769	0.5423
Cereal-WB:Protein-LP:Butyrate-But	-0.1200	0.1162	-0.0588	0.7775
Insulin receptor β				
	Gene expression		**Protein abundance**	
	Estimate	**SE**	**Estimate**	**SE**
Intercept	0.1485	0.0444	0.7394	0.0791
Cereal-WB	-0.0808	0.0645	0.7673	0.1118
Protein-LP	-0.0163	0.0645	-0.0206	0.1090
Butyrate-But	-0.0766	0.0645	-0.2766	0.1090
Cereal-WB:Protein-LP	0.2426	0.0924	-0.5054	0.1541
Cereal-WB:Butyrate-But	0.2230	0.0924	-0.0714	0.1541
Protein-LP:Butyrate-But	0.0791	0.0912	0.2660	0.1521
Cereal-WB:Protein-LP:Butyrate-But	-0.4093	0.1317	0.2324	0.2151
Mammalian target of rapamycin				
	Gene expression		**Protein abundance**	
	Estimate	**SE**	**Estimate**	**SE**
Intercept	0.0035	0.0013	1.1869	0.4277
Cereal-WB	0.0013	0.0017	0.7111	0.6215
Protein-LP	0.0037	0.0017	0.1169	0.6049
Butyrate-But	0.0015	0.0017	-0.4869	0.6416
Cereal-WB:Protein-LP	0.0025	0.0024	0.1240	0.8672
Cereal-WB:Butyrate-But	-0.0010	0.0024	0.8517	0.8932
Protein-LP:Butyrate-But	-0.0022	0.0024	0.9313	0.8818
Cereal-WB:Protein-LP:Butyrate-But	-0.0015	0.0033	-0.5158	1.2368

WB: Wheat based diet supplemented with NSP-degrading xylanase and glucanase enzymes; LP: “Low protein” group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); SE: Standard error of the estimate. Nutritional factors with colon in between show interactions (Cereal:

Cereal type; Protein: Crude protein level; Butyrate: Butyrate supplementation). Model fitting was performed with the *lm* built-in function of the R statistical programming environment (v4.0.3).
n = 10 per group.

Table S2. ANOVA analysis results of the gene expression and protein abundance data for each analyzed signaling element.

Glucagon receptor				
	Gene expression		**Protein abundance**	
	F statistics	**P value**	**F statistics**	**P value**
Cereal type	7.8132	0.0067*	2.4425	0.1226
Crude protein level	12.5253	0.0007*	0.5888	0.4455
Butyrate supplement.	0.0082	0.9279	5.4513	0.0224*
Cereal:Protein	1.2630	0.2650	1.8139	0.1824
Cereal:Butyrate	0.0134	0.9083	5.4408	0.0226*
Protein:Butyrate	1.5586	0.2162	5.9535	0.0172*
Cereal:Protein:Butyrate	1.0672	0.3052	0.0057	0.9399
Insulin receptor β				
	Gene expression		**Protein abundance**	
	F statistics	**P value**	**F statistics**	**P value**
Cereal type	2.1432	0.1480	97.7768	<0.0001*
Crude protein level	1.7829	0.1865	2.0532	0.1563
Butyrate supplement.	0.6197	0.4340	4.6587	0.0343*
Cereal:Protein	0.3916	0.5336	12.8919	0.0006*
Cereal:Butyrate	0.1074	0.7442	0.1993	0.6567
Protein:Butyrate	3.1747	0.0795	12.6278	0.0007*
Cereal:Protein:Butyrate	9.6671	0.0028*	1.1677	0.2836
Mammalian target of rapamycin				
	Gene expression		**Protein abundance**	
	F statistics	**P value**	**F statistics**	**P value**
Cereal type	4.1528	0.0456*	11.7942	0.0010*
Crude protein level	17.4478	<0.0001*	2.6624	0.1073
Butyrate supplement.	0.3849	0.5371	0.9219	0.3403
Cereal:Protein	1.1052	0.2970	0.0440	0.8346
Cereal:Butyrate	1.1016	0.2977	0.8895	0.3489
Protein:Butyrate	3.1432	0.0809	1.1711	0.2829
Cereal:Protein:Butyrate	0.1997	0.6565	0.1739	0.6780

F statistics and P values are given in the corresponding columns. The study was conducted with two types of cereal (wheat-based diet supplemented with NSP-degrading xylanase and glucanase enzymes [WB] or maize-based diet [MB]), normal (NP) or lowered crude protein level (LP; reduced by 15 %, supplemented with limiting amino acids), and with or without sodium (n-)butyrate supplementation (1.5 g/kg diet). Main effects were determined as follows: WB vs. MB diet (Cereal type), LP vs. NP groups (Crude protein level) and butyrate supplementation vs. no added butyrate (Butyrate supplement.). Nutritional factors with colon in between show interactions (Cereal: Cereal type; Protein: Crude protein level; Butyrate: Butyrate supplement.). Asterisk (*) indicates statistically significant effects ($P < 0.05$). Calculations were performed with the *Anova* function in the *car* package of the R statistical programming environment (v4.0.3).
n = 10 per group.

Table S3: *Post hoc* pairwise comparisons of the different treatment groups of the glucagon receptor gene expression data.

Contrast	Estimate	SE	CI	DF	t value	P value
MB NP Ctr – MB LP Ctr	-0.0773	0.0581	(-0.2589, 0.1044)	68	-1.330	0.8841
MB NP Ctr – WB NP Ctr	-0.0195	0.0596	(-0.2059, 0.1669)	68	-0.327	>0.9999
MB NP Ctr – WB LP Ctr	-0.2236	0.0596	(-0.4100, -0.0372)	68	-3.752	0.0083*
MB NP Ctr – MB NP But	-0.0116	0.0596	(-0.1980, 0.1748)	68	-0.195	>0.9999
MB NP Ctr – MB LP But	-0.0764	0.0581	(-0.2580, 0.1053)	68	-1.315	0.8902
MB NP Ctr – WB NP But	-0.0860	0.0581	(-0.2677, 0.0957)	68	-1.480	0.8150
MB NP Ctr – WB LP But	-0.1576	0.0581	(-0.3393, 0.0241)	68	-2.713	0.1366
MB LP Ctr – WB NP Ctr	0.0578	0.0581	(-0.1239, 0.2394)	68	0.994	0.9738
MB LP Ctr – WB LP Ctr	-0.1464	0.0581	(-0.3280, 0.0353)	68	-2.519	0.2051
MB LP Ctr – MB NP But	0.0656	0.0581	(-0.1160, 0.2473)	68	1.130	0.9479
MB LP Ctr – MB LP But	0.0009	0.0565	(-0.1759, 0.1777)	68	0.016	>0.9999
MB LP Ctr – WB NP But	-0.0087	0.0565	(-0.1856, 0.1681)	68	-0.155	>0.9999
MB LP Ctr – WB LP But	-0.0803	0.0565	(-0.2571, 0.0965)	68	-1.420	0.8447
WB NP Ctr – WB LP Ctr	-0.2041	0.0596	(-0.3905, -0.0177)	68	-3.425	0.0221*
WB NP Ctr – MB NP But	0.0079	0.0596	(-0.1785, 0.1943)	68	0.132	>0.9999
WB NP Ctr – MB LP But	-0.0569	0.0581	(-0.2385, 0.1248)	68	-0.979	0.9759
WB NP Ctr – WB NP But	-0.0665	0.0581	(-0.2482 , 0.1152)	68	-1.145	0.9442
WB NP Ctr – WB LP But	-0.1381	0.0581	(-0.3197, 0.0436)	68	-2.377	0.2694
WB LP Ctr – MB NP But	0.2120	0.0596	(0.0256, 0.3984)	68	3.557	0.0150*
WB LP Ctr – MB LP But	0.1473	0.0581	(-0.0344, 0.3289)	68	2.535	0.1989
WB LP Ctr – WB NP But	0.1376	0.0581	(-0.0440, 0.3193)	68	2.369	0.2733
WB LP Ctr – WB LP But	0.0660	0.0581	(-0.1156, 0.2477)	68	1.137	0.9462
MB NP But – MB LP But	-0.0648	0.0581	(-0.2464, 0.1169)	68	-1.115	0.9514
MB NP But – WB NP But	-0.0744	0.0581	(-0.2561, 0.1073)	68	-1.280	0.9030
MB NP But – WB LP But	-0.1460	0.0581	(-0.3276, 0.0357)	68	-2.513	0.2080
MB LP But – WB NP But	-0.0096	0.0565	(-0.1865, 0.1672)	68	-0.170	>0.9999
MB LP But – WB LP But	-0.0812	0.0565	(-0.2580, 0.0956)	68	-1.436	0.8371
WB NP But – WB LP But	-0.0716	0.0565	(-0.2484, 0.1052)	68	-1.266	0.9081

MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: “Normal protein” group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: “Low protein” group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation. The calculated difference of the estimated marginal means of the treatment groups compared (Contrast) can be found in the column Estimate, alongside with its standard error and confidence interval (columns SE and CI, respectively). Degrees of freedom (DF), t value and P values can be found in their corresponding columns. Asterisk (*) indicates statistically significant differences ($P < 0.05$). Pairwise comparisons were performed by the *emmeans* package of the R statistical programming environment (v4.0.3), P values and confidence intervals were adjusted with the Tukey method.

Table S4: *Post hoc* pairwise comparisons of the different treatment groups of the glucagon receptor protein abundance data.

Contrast	Estimate	SE	CI	DF	t value	P value
MB NP Ctr – MB LP Ctr	0.3828	0.383	(-0.815, 1.581)	70	0.998	0.9732
MB NP Ctr – WB NP Ctr	-1.0107	0.394	(-2.242, 0.220)	70	-2.565	0.1864
MB NP Ctr – WB LP Ctr	-0.1337	0.383	(-1.332, 1.064)	70	-0.349	>0.9999
MB NP Ctr – MB NP But	0.5123	0.383	(-0.686, 1.711)	70	1.336	0.8818
MB NP Ctr – MB LP But	-0.0817	0.383	(-1.280, 1.116)	70	-0.213	>0.9999
MB NP Ctr – WB NP But	0.3779	0.394	(-0.853, 1.609)	70	0.959	0.9786
MB NP Ctr – WB LP But	0.3369	0.383	(-0.861, 1.535)	70	0.878	0.9871
MB LP Ctr – WB NP Ctr	-1.3935	0.394	(-2.625, -0.163)	70	-3.537	0.0157*
MB LP Ctr – WB LP Ctr	-0.5165	0.383	(-1.715, 0.682)	70	-1.347	0.8774
MB LP Ctr – MB NP But	0.1295	0.383	(-1.069, 1.328)	70	0.338	>0.9999
MB LP Ctr – MB LP But	-0.4645	0.383	(-1.663, 0.734)	70	-1.211	0.9259
MB LP Ctr – WB NP But	-0.0049	0.394	(-1.236, 1.226)	70	-0.012	>0.9999
MB LP Ctr – WB LP But	-0.0459	0.383	(-1.244, 1.152)	70	-0.120	>0.9999
WB NP Ctr – WB LP Ctr	0.8770	0.394	(-0.354, 2.108)	70	2.226	0.3494
WB NP Ctr – MB NP But	1.5231	0.394	(0.292, 2.754)	70	3.866	0.0057*
WB NP Ctr – MB LP But	0.9290	0.394	(-0.302, 2.160)	70	2.358	0.2783
WB NP Ctr – WB NP But	1.3886	0.404	(0.126, 2.652)	70	3.435	0.0212*
WB NP Ctr – WB LP But	1.3476	0.394	(0.117, 2.579)	70	3.420	0.0221*
WB LP Ctr – MB NP But	0.6461	0.383	(-0.552, 1.844)	70	1.685	0.6969
WB LP Ctr – MB LP But	0.0520	0.383	(-1.146, 1.250)	70	0.136	>0.9999
WB LP Ctr – WB NP But	0.5116	0.394	(-0.719, 1.743)	70	1.299	0.8964
WB LP Ctr – WB LP But	0.4706	0.383	(-0.728, 1.669)	70	1.227	0.9210
MB NP But – MB LP But	-0.5941	0.383	(-1.792, 0.604)	70	-1.549	0.7780
MB NP But – WB NP But	-0.1344	0.394	(-1.365, 1.097)	70	-0.341	>0.9999
MB NP But – WB LP But	-0.1755	0.383	(-1.374, 1.023)	70	-0.458	0.9998
MB LP But – WB NP But	0.4596	0.394	(-0.771, 1.691)	70	1.167	0.9386
MB LP But – WB LP But	0.4186	0.383	(-0.780, 1.617)	70	1.092	0.9565
WB NP But – WB LP But	-0.0411	0.394	(-1.272, 1.190)	70	-0.104	>0.9999

MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: “Normal protein” group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: “Low protein” group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation. The calculated difference of the estimated marginal means of the treatment groups compared (Contrast) can be found in the column Estimate, alongside with its standard error and confidence interval (columns SE and CI, respectively). Degrees of freedom (DF), t value and P values can be found in their corresponding columns. Asterisk (*) indicates statistically significant differences ($P < 0.05$). Pairwise comparisons were performed by the *emmeans* package of the R statistical programming environment (v4.0.3), P values and confidence intervals were adjusted with the Tukey method.

Table S5: *Post hoc* pairwise comparisons of the different treatment groups of the insulin receptor β gene expression data.

Contrast	Estimate	SE	CI	DF	t value	P value
MB NP Ctr – MB LP Ctr	0.0163	0.0645	(-0.1856, 0.2181)	65	0.252	>0.9999
MB NP Ctr – WB NP Ctr	0.0808	0.0645	(-0.1210, 0.2827)	65	1.254	0.9120
MB NP Ctr – WB LP Ctr	-0.1455	0.0645	(-0.3474, 0.0563)	65	-2.258	0.3326
MB NP Ctr – MB NP But	0.0766	0.0645	(-0.1253, 0.2785)	65	1.189	0.9324
MB NP Ctr – MB LP But	0.0138	0.0627	(-0.1827, 0.2103)	65	0.220	>0.9999
MB NP Ctr – WB NP But	-0.0655	0.0645	(-0.2674, 0.1363)	65	-1.017	0.9702
MB NP Ctr – WB LP But	0.0383	0.0665	(-0.1701, 0.2467)	65	0.576	0.9991
MB LP Ctr – WB NP Ctr	0.0646	0.0661	(-0.1425, 0.2717)	65	0.977	0.9762
MB LP Ctr – WB LP Ctr	-0.1619	0.0661	(-0.3689, 0.0453)	65	-2.446	0.2374
MB LP Ctr – MB NP But	0.0604	0.0661	(-0.1467, 0.2675)	65	0.913	0.9838
MB LP Ctr – MB LP But	-0.0025	0.0645	(-0.2043, 0.1994)	65	-0.038	>0.9999
MB LP Ctr – WB NP But	-0.0818	0.0661	(-0.2889, 0.1253)	65	-1.237	0.9177
MB LP Ctr – WB LP But	0.0221	0.0682	(-0.1914, 0.2356)	65	0.324	>0.9999
WB NP Ctr – WB LP Ctr	-0.2264	0.0661	(-0.4335, -0.0192)	65	-3.423	0.0226*
WB NP Ctr – MB NP But	-0.0042	0.0661	(-0.2113, 0.2029)	65	-0.064	>0.9999
WB NP Ctr – MB LP But	-0.0671	0.0645	(-0.2689, 0.1348)	65	-1.040	0.9663
WB NP Ctr – WB NP But	-0.1464	0.0661	(-0.3535, 0.0607)	65	-2.213	0.3577
WB NP Ctr – WB LP But	-0.0425	0.0682	(-0.2560, 0.1710)	65	-0.624	0.9984
WB LP Ctr – MB NP But	0.2222	0.0661	(0.0150, 0.4293)	65	3.359	0.0270*
WB LP Ctr – MB LP But	0.1593	0.0645	(-0.0426, 0.3612)	65	2.472	0.2261
WB LP Ctr – WB NP But	0.0800	0.0661	(-0.1271, 0.2871)	65	1.210	0.9262
WB LP Ctr – WB LP But	0.1839	0.0682	(-0.0296, 0.3973)	65	2.697	0.1421
MB NP But – MB LP But	-0.0628	0.0645	(-0.2647, 0.1390)	65	-0.975	0.9765
MB NP But – WB NP But	-0.1422	0.0661	(-0.3493, 0.0650)	65	-2.150	0.3954
MB NP But – WB LP But	-0.0383	0.0682	(-0.2518, 0.1752)	65	-0.562	0.9992
MB LP But – WB NP But	-0.0793	0.0645	(-0.2812, 0.1225)	65	-1.231	0.9197
MB LP But – WB LP But	0.0245	0.0665	(-0.1839, 0.2329)	65	0.369	>0.9999
WB NP But – WB LP But	0.1039	0.0682	(-0.1096, 0.3174)	65	1.524	0.7919

MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: “Normal protein” group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: “Low protein” group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation. The calculated difference of the estimated marginal means of the treatment groups compared (Contrast) can be found in the column Estimate, alongside with its standard error and confidence interval (columns SE and CI, respectively). Degrees of freedom (DF), t value and P values can be found in their corresponding columns. Asterisk (*) indicates statistically significant differences ($P < 0.05$). Pairwise comparisons were performed by the *emmeans* package of the R statistical programming environment (v4.0.3), P values and confidence intervals were adjusted with the Tukey method.

Table S6: *Post hoc* pairwise comparisons of the different treatment groups of the insulin receptor β protein abundance data.

Contrast	Estimate	SE	CI	DF	t value	P value
MB NP Ctr – MB LP Ctr	0.0206	0.109	(-0.3199, 0.3611)	70	0.189	>0.9999
MB NP Ctr – WB NP Ctr	-0.7673	0.112	(-1.1167, -0.4180)	70	-6.862	<.0001*
MB NP Ctr – WB LP Ctr	-0.2414	0.109	(-0.5819, 0.0992)	70	-2.215	0.3560
MB NP Ctr – MB NP But	0.2766	0.109	(-0.0639, 0.6171)	70	2.538	0.1972
MB NP Ctr – MB LP But	0.0312	0.109	(-0.3093, 0.3718)	70	0.287	>0.9999
MB NP Ctr – WB NP But	-0.4194	0.109	(-0.7599, -0.0788)	70	-3.848	0.0060*
MB NP Ctr – WB LP But	-0.3918	0.109	(-0.7324, -0.0513)	70	-3.595	0.0132*
MB LP Ctr – WB NP Ctr	-0.7879	0.109	(-1.1285, -0.4474)	70	-7.229	<.0001*
MB LP Ctr – WB LP Ctr	-0.2620	0.106	(-0.5934, 0.0695)	70	-2.469	0.2258
MB LP Ctr – MB NP But	0.2560	0.106	(-0.0755, 0.5875)	70	2.413	0.2514
MB LP Ctr – MB LP But	0.0106	0.106	(-0.3208, 0.3421)	70	0.100	>0.9999
MB LP Ctr – WB NP But	-0.4400	0.106	(-0.7714, -0.1085)	70	-4.147	0.0023*
MB LP Ctr – WB LP But	-0.4124	0.106	(-0.7439, -0.0809)	70	-3.887	0.0053*
WB NP Ctr – WB LP Ctr	0.5260	0.109	(0.1854, 0.8665)	70	4.826	0.0002*
WB NP Ctr – MB NP But	1.0439	0.109	(0.7034, 1.3845)	70	9.578	<.0001*
WB NP Ctr – MB LP But	0.7986	0.109	(0.4580, 1.1391)	70	7.327	<.0001*
WB NP Ctr – WB NP But	0.3480	0.109	(0.0074, 0.6885)	70	3.193	0.0417*
WB NP Ctr – WB LP But	0.3755	0.109	(0.0350, 0.7161)	70	3.445	0.0206*
WB LP Ctr – MB NP But	0.5180	0.106	(0.1865, 0.8494)	70	4.883	0.0002*
WB LP Ctr – MB LP But	0.2726	0.106	(-0.0589, 0.6041)	70	2.570	0.1848
WB LP Ctr – WB NP But	-0.1780	0.106	(-0.5095, 0.1535)	70	-1.678	0.7011
WB LP Ctr – WB LP But	-0.1504	0.106	(-0.4819, 0.1810)	70	-1.418	0.8459
MB NP But – MB LP But	-0.2454	0.106	(-0.5768, 0.0861)	70	-2.313	0.3015
MB NP But – WB NP But	-0.6960	0.106	(-1.0274, -0.3645)	70	-6.561	<.0001*
MB NP But – WB LP But	-0.6684	0.106	(-0.9999, -0.3369)	70	-6.301	<.0001*
MB LP But – WB NP But	-0.4506	0.106	(-0.7821, -0.1192)	70	-4.248	0.0016*
MB LP But – WB LP But	-0.4230	0.106	(-0.7545, -0.0916)	70	-3.988	0.0038*
WB NP But – WB LP But	0.0276	0.106	(-0.3039, 0.3590)	70	0.260	>0.9999

MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: “Normal protein” group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: “Low protein” group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation. The calculated difference of the estimated marginal means of the treatment groups compared (Contrast) can be found in the column Estimate, alongside with its standard error and confidence interval (columns SE and CI, respectively). Degrees of freedom (DF), t value and P values can be found in their corresponding columns. Asterisk (*) indicates statistically significant differences ($P < 0.05$). Pairwise comparisons were performed by the *emmeans* package of the R statistical programming environment (v4.0.3), P values and confidence intervals were adjusted with the Tukey method.

Table S7: *Post hoc* pairwise comparisons of the different treatment groups of the mammalian target of rapamycin gene expression data.

Contrast	Estimate	SE	CI	DF	t value	P value
MB NP Ctr – MB LP Ctr	-0.0037	0.00174	(-0.0092, 0.0017)	66	-2.146	0.3977
MB NP Ctr – WB NP Ctr	-0.0013	0.00174	(-0.0068, 0.0041)	66	-0.756	0.9947
MB NP Ctr – WB LP Ctr	-0.0076	0.00174	(-0.0130, -0.0021)	66	-4.360	0.0012*
MB NP Ctr – MB NP But	-0.0015	0.00174	(-0.0069, 0.0040)	66	-0.860	0.9885
MB NP Ctr – MB LP But	-0.0030	0.00170	(-0.0084, 0.0023)	66	-1.789	0.6296
MB NP Ctr – WB NP But	-0.0018	0.00170	(-0.0071, 0.0035)	66	-1.076	0.9597
MB NP Ctr – WB LP But	-0.0044	0.00170	(-0.0097, 0.0009)	66	-2.600	0.1745
MB LP Ctr – WB NP Ctr	0.0024	0.00169	(-0.0029, 0.0077)	66	1.433	0.8387
MB LP Ctr – WB LP Ctr	-0.0039	0.00169	(-0.0091, 0.0014)	66	-2.283	0.3185
MB LP Ctr – MB NP But	0.0022	0.00169	(-0.0030, 0.0075)	66	1.325	0.8860
MB LP Ctr – MB LP But	0.0007	0.00165	(-0.0045, 0.0058)	66	0.422	0.9999
MB LP Ctr – WB NP But	0.0019	0.00165	(-0.0032, 0.0071)	66	1.158	0.9406
MB LP Ctr – WB LP But	-0.0007	0.00165	(-0.0058, 0.0045)	66	-0.415	0.9999
WB NP Ctr – WB LP Ctr	-0.0063	0.00169	(-0.0116, -0.0010)	66	-3.716	0.0094*
WB NP Ctr – MB NP But	-0.0002	0.00169	(-0.0055, 0.0051)	66	-0.108	>0.9999
WB NP Ctr – MB LP But	-0.0017	0.00165	(-0.0069, 0.0034)	66	-1.048	0.9650
WB NP Ctr – WB NP But	-0.0005	0.00165	(-0.0057, 0.0046)	66	-0.312	>0.9999
WB NP Ctr – WB LP But	-0.0031	0.00165	(-0.0083, 0.0020)	66	-1.885	0.5657
WB LP Ctr – MB NP But	0.0061	0.00169	(0.0008, 0.0114)	66	3.608	0.0131*
WB LP Ctr – MB LP But	0.0045	0.00165	(-0.0006, 0.0097)	66	2.764	0.1221
WB LP Ctr – WB NP But	0.0058	0.00165	(0.0006, 0.0109)	66	3.501	0.0179*
WB LP Ctr – WB LP But	0.0032	0.00165	(-0.0020, 0.0083)	66	1.927	0.5379
MB NP But – MB LP But	-0.0015	0.00165	(-0.0067, 0.0036)	66	-0.937	0.9811
MB NP But – WB NP But	-0.0003	0.00165	(-0.0055, 0.0048)	66	-0.201	>0.9999
MB NP But – WB LP But	-0.0029	0.00165	(-0.0081, 0.0022)	66	-1.775	0.6391
MB LP But – WB NP But	0.0012	0.00160	(-0.0038, 0.0062)	66	0.757	0.9947
MB LP But – WB LP But	-0.0014	0.00160	(-0.0064, 0.0036)	66	-0.860	0.9885
WB NP But – WB LP But	-0.0026	0.00160	(-0.0076, 0.0024)	66	-1.617	0.7386

MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: “Normal protein” group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: “Low protein” group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation. The calculated difference of the estimated marginal means of the treatment groups compared (Contrast) can be found in the column Estimate, alongside with its standard error and confidence interval (columns SE and CI, respectively). Degrees of freedom (DF), t value and P values can be found in their corresponding columns. Asterisk (*) indicates statistically significant differences ($P < 0.05$). Pairwise comparisons were performed by the *emmeans* package of the R statistical programming environment (v4.0.3), P values and confidence intervals were adjusted with the Tukey method.

Table S8: *Post hoc* pairwise comparisons of the different treatment groups of the mammalian target of rapamycin protein abundance data.

Contrast	Estimate	SE	CI	DF	t value	P value
MB NP Ctr – MB LP Ctr	-0.117	0.605	(-2.008, 1.774)	69	-0.193	>0.9999
MB NP Ctr – WB NP Ctr	-0.711	0.621	(-2.654, 1.232)	69	-1.144	0.9444
MB NP Ctr – WB LP Ctr	-0.952	0.605	(-2.843, 0.939)	69	-1.574	0.7640
MB NP Ctr – MB NP But	0.487	0.642	(-1.519, 2.492)	69	0.759	0.9946
MB NP Ctr – MB LP But	-0.561	0.605	(-2.452, 1.330)	69	-0.928	0.9823
MB NP Ctr – WB NP But	-1.076	0.605	(-2.967, 0.815)	69	-1.779	0.6363
MB NP Ctr – WB LP But	-1.732	0.605	(-3.623, 0.159)	69	-2.864	0.0963
MB LP Ctr – WB NP Ctr	-0.594	0.621	(-2.537, 1.348)	69	-0.956	0.9790
MB LP Ctr – WB LP Ctr	-0.835	0.605	(-2.726, 1.056)	69	-1.380	0.8630
MB LP Ctr – MB NP But	0.604	0.642	(-1.402, 2.609)	69	0.941	0.9808
MB LP Ctr – MB LP But	-0.444	0.605	(-2.335, 1.446)	69	-0.735	0.9956
MB LP Ctr – WB NP But	-0.959	0.605	(-2.850, 0.932)	69	-1.585	0.7572
MB LP Ctr – WB LP But	-1.615	0.605	(-3.506, 0.275)	69	-2.671	0.1495
WB NP Ctr – WB LP Ctr	-0.241	0.621	(-2.183, 1.702)	69	-0.388	0.9999
WB NP Ctr – MB NP But	1.198	0.657	(-0.856, 3.252)	69	1.823	0.6071
WB NP Ctr – MB LP But	0.150	0.621	(-1.793, 2.092)	69	0.241	>0.9999
WB NP Ctr – WB NP But	-0.365	0.621	(-2.307, 1.578)	69	-0.587	0.9989
WB NP Ctr – WB LP But	-1.021	0.621	(-2.964, 0.921)	69	-1.643	0.7227
WB LP Ctr – MB NP But	1.439	0.642	(-0.567, 3.444)	69	2.243	0.3402
WB LP Ctr – MB LP But	0.391	0.605	(-1.500, 2.281)	69	0.646	0.9980
WB LP Ctr – WB NP But	-0.124	0.605	(-2.015, 1.767)	69	-0.205	>0.9999
WB LP Ctr – WB LP But	-0.780	0.605	(-2.671, 1.110)	69	-1.290	0.8995
MB NP But – MB LP But	-1.048	0.642	(-3.054, 0.957)	69	-1.634	0.7284
MB NP But – WB NP But	-1.563	0.642	(-3.568, 0.443)	69	-2.436	0.2411
MB NP But – WB LP But	-2.219	0.642	(-4.225, -0.214)	69	-3.459	0.0199*
MB LP But – WB NP But	-0.515	0.605	(-2.405, 1.376)	69	-0.851	0.9893
MB LP But – WB LP But	-1.171	0.605	(-3.062, 0.720)	69	-1.936	0.5318
WB NP But – WB LP But	-0.656	0.605	(-2.547, 1.234)	69	-1.085	0.9579

MB: Maize-based diet; WB: Wheat-based diet completed with NSP-degrading xylanase and glucanase enzymes; NP: “Normal protein” group with dietary crude protein content adequate to the rearing phase (22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets); LP: “Low protein” group with reduced crude protein content (19.1%, 18.0% and 16.0% crude protein, respectively), supplemented with limiting amino acids; But: Sodium butyrate supplementation of the diet (1.5 g/kg diet); Ctr: Control group without sodium butyrate supplementation. The calculated difference of the estimated marginal means of the treatment groups compared (Contrast) can be found in the column Estimate, alongside with its standard error and confidence interval (columns SE and CI, respectively). Degrees of freedom (DF), t value and P values can be found in their corresponding columns. Asterisk (*) indicates statistically significant differences ($P < 0.05$). Pairwise comparisons were performed by the *emmeans* package of the R statistical programming environment (v4.0.3), P values and confidence intervals were adjusted with the Tukey method.

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