Fine characterization and microbiota assessment as keys to understanding the positive effect of standardized natural citrus extract on broiler chickens

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FINE CHARACTERIZATION AND MICROBIOTA ASSESSMENT AS KEYS TO UNDERSTANDING THE POSITIVE EFFECT OF STANDARDIZED NATURAL CITRUS EXTRACT ON BROILER CHICKENS[1]

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LAY SUMMARY

Citrus extracts are increasingly being used in animal nutrition to enhance animal growth performances. Most of the available studies indicate an effect of these extracts on microbiota. However, citrus extracts can vary a lot. Indeed, the composition of citrus extract depends on parameters such as the citrus species, the extraction methods, and the inclusion rate. This variation is very important to take into consideration before using a citrus extract. The objective here was to evaluate a commercially available standardized natural citrus extract in terms of composition and effect on broiler chickens' performances and microbiota. Results showed that standardized natural citrus extract positively affects the final weight of broilers, but no effect was observed on chickens' caecal microbiota. The characterization of the standardized natural citrus extract reveals pectic oligosaccharides as major compounds as well as 35 others molecules. Most of these compounds are well described for their beneficial effect on animals' performances and health. In conclusion, the standardized natural citrus extract showed beneficial effects on broilers' performances. These effects are not correlated with broilers microbiota modulation and may be explained by the composition of the product.

TEASER TEXT

Standardized natural citrus extract positively influences broilers growth performances without any effect on microbiota.

Characterization of standardized natural citrus extract allows to evidence its standardization and to set up hypotheses regarding its mode of action.

ABSTRACT

The objective of this study was to investigate the effect and composition of a standardized natural citrus extract (**SNCE**) on both broiler chickens' growth performances and intestinal microbiota. A total of 930 one-day-old males were randomly assigned to 3 dietary treatments: a control treatment (**CTL**) in which broiler chickens were fed with a standard diet and 2 citrus treatments in which broiler chickens were fed with the same standard diet supplemented with 250 ppm and 2,500 ppm of SNCE, respectively. Each dietary treatment was composed of 10 experimental units (pen) of 31 broiler chickens each. Growth performances such as feed consumption, body weight, and feed conversion ratio (**FCR**) were recorded weekly until d 42. Litter quality was also weekly recorded while mortality was daily recorded. One broiler chicken was randomly selected from each pen (10 chickens/group) and ceca samples were collected for microbiota analysis at d 7 and 42. Chromatographic methods were used to determine molecules that enter into the composition of the SNCE. Results from the characterization of SNCE allowed to identify pectic oligosaccharides (**POS**) as a major component of the SNCE. In addition, 35 secondary metabolites, including eriocitrin, hesperidin, and naringin, were identified. The experiment performed on broiler chickens showed that the final body weight of broiler chickens fed diets supplemented with SNCE was higher than those fed the CTL diets ($P < 0.01$). Broiler cecal microbiota was impacted by age ($P < 0.01$) but not by the dietary supplementation of SNCE. Results indicate that SNCE allowed enhancing chickens' performances without any modulation of the cecal microbiota of broiler chickens. The characterization of SNCE allowed to identify compounds such as eriocitrin, naringin, hesperidin, and POS. Thus, opening new horizons for a better understanding of the observed effect on broiler chickens' growth performances.

Keywords: citrus composition, citrus extract, growth performance, gut, microbiota.

LIST OF ABBREVIATION

AGP: Antibiotic growth promotors

ANAN: analyse des acides nucléiques

BIBS : Bioressources, imagerie, biochimie et structure

BSTFA: Bis(trimethylsilyl)trifluoroacetamide

CTL: control

ELSD: Evaporative light scattering detector

FCR: feed conversion ration

GC-FID: Gaz chromatography- Flame ionization detector

GC-MS: Gaz chromatography – mass spectrometry

HPLC: High performances liquid chromatography

ITAVI: French technical poultry institute

MHDP: meta-hydroxyphenyl

MDS: Multidimensional scaling

nMDS: non-metric multidimensional scaling

PCR: polymerase chain reaction

POS: pectic oligosaccharides

SNCE: Standardized natural citrus extract

INTRODUCTION

The ban of antibiotic growth promoters (**AGP**) in the European Union was a turning point in the field of animal health and nutrition (Castanon, 2007). In fact, the number of alternatives to AGPs increased considerably during this period, aiming to achieve a similar beneficial effect on growth performance (Verstegen and Williams, 2002). These alternatives include but are not limited to exogenous enzymes, organic acids, prebiotics, probiotics, and plant extracts (Huyghebaert et al., 2011). Nowadays, most of these alternatives have proved their effectiveness in the field (Ebrahimi et al., 2015; Puvača et al., 2013; Selle et al., 2009). However, the observed effect of these alternatives can sometimes vary over time. This is particularly true for alternatives based on plants and/or plant extracts. These variabilities may be explained by many factors such as the manufacturing process, the origin, and the poor and/or absence of standardization.

In this study, we focused on citrus extracts which are particularly studied with variable effects on broiler chickens' growth performances and health. Some studies showed beneficial effects on broiler chickens 'growth performances (Abbasi et al., 2015; Boumezrag et al., 2018; Juin et al., 2003; Seidavi et al., 2018). However, other studies do not report beneficial effects of citrus extract dietary supplementation on broiler chickens' growth performances (Akbarian et al., 2013; Alzawqari et al., 2016). The differences in the reported effects may be partly explained by the high variability of the extracts in terms of composition and concentration of active compounds. However, most of the citrus products show no or very limited composition data, which is an obstacle to the understanding of their mechanism of action. To determine viable long-term alternatives, it is necessary to go beyond performance and look to characterize and standardize citrus extract-based alternatives.

The objective of this study was to obtain some key elements to understand/explain the effect of dietary supplementation of citrus extract on broiler chickens. This was done first by fine characterization of the citrus extract (Nor-Spice AB®, Nor Feed SAS, France) and followed by the *in*

vivo effect on broiler chickens' growth performances. Finally, the possible effects on cecal microbiota composition were also investigated.

MATERIAL AND METHODS

This study was carried out in strict accordance with the recommendations set out in the European Guidelines for accommodation and care of animals (Directive 2010/63/EU revising Directive 86/609/EEC).

Samples, animals, and diet

Standardized natural citrus extract (**SNCE**, Nor-Spice AB) was supplied by Nor-Feed SAS Company (France). It is a 100% natural feed additive based on citrus extract and standardized in terms of citroflavonoids (≥ 6%). Nine hundred thirty 1-d-old males (Ross 308, Aviagen, Beaucouze, Maine et Loire, France) were randomly assigned to 3 dietary treatments: a control treatment (**CTL**) in which broiler chickens were fed with a standard diet and 2 citrus treatments, in which broiler chickens were respectively fed with standard diet supplemented with 250 ppm (**SNCE 250**) and 2,500 ppm (**SNCE 2,500**) of SNCE. The feeding program was set up as follows: starter from d 1 to d 14, grower from d 14 to d 28, and finisher from d 28 until the end. Nutritional values of the 3 diets are described in Table 1 and 2. Each treatment was composed of 10 repetitions pens of 31 broiler chickens per pen. Broiler chickens were reared until d 42.

Broiler chickens were fed using a hung pan feeder system (one per pen) and a dedicated nipple line was used for water (ad libitum). The following prophylaxis program was set up: d 0: infectious bronchitis vaccine (aerosol spraying) and d 17: infectious bursal disease vaccine (drinking water). No other treatment was applied during the trial. Wood dust was used for litter. The lighting program was set up as follows: d 0 to d 4: 24 h a day of lighting with 100 % light intensity, d 5 to d 42: 18 h a day of lighting with 30 to 40% light intensity. Feed consumption and body weight per pen were recorded weekly from d 14 to d 42. Feed conversion ratio (**FCR**) was also weekly calculated from d 14

to d 42. Mortality was recorded daily, and litter quality was evaluated at d 14, d 21, d 28, d 35, and d 42 following french technical poultry institute (**ITAVI**) procedure for scoring (Bignon et al., 2015).

Sequencing

Ten broiler chickens per treatment (1 per pen) were randomly selected and humanly euthanized at d 7 and d 42 and ceca contents were sampled for microbiota analysis. Samples were kept in an airtight jar at -80°C until use. Libraries construction, DNA extraction, and sequencing were performed at the Analyse des acides nucléiques platform (ANAN, Structure fédérative de la recherche Qualité et Santé du végétal, Angers, France). DNA samples were extracted and purified using the ZR-96 Soil Microbe DNA kit (Zymo Research, Irvine, CA, USA). Illumina sequencing libraries were prepared according to Caporaso et al. (Caporaso et al., 2012). A first Polymerase Chain Reaction (**PCR**) amplification was performed with the primer sets 515f/806r (Caporaso et al., 2012), which target the V4 region of the 16 rRNA gene. PCR reactions were performed in 50 µL with a high-fidelity Taq DNA polymerase (AccuPrime^{TM} Taq DNA Polymerase System, Invitrogen, USA) using 5µL of 10X Buffer, 1µL of forward primer, and 1 µL of reverse primer at 10µM each, 0.2µL of Taq, and 5 µl of DNA.

Cycling conditions were composed of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of amplification at 94°C (30 s), 50°C (45 s), and 68°C (90 s), and final elongation at 68°C for 10 min. Amplicons were purified with magnetic beads (NucleoMag NGS Clean-up and Size Select, Macherey-Nagel, Düren, Germany). Second PCR amplification was performed to incorporate Illumina adapters and barcodes. PCR cycling conditions consists of a first denaturation at 94°C (1 min), followed by 12 cycles at 94°C (1min), 55°C (1 min), and 68°C (1 min), and final elongation at 68°C for 10 min. Amplicons were purified with magnetic beads. All the amplicons were pooled and the concentration of the pool was monitored with quantitative PCR (KAPA Library Quantification Kit, Roche, Basel, Switzerland). Amplicon libraries were mixed with 5% PhiX and sequenced with a MiSeq reagent kit v2 500 cycles (Illumina, San Diego California, USA). Then, each sequence was assigned to its sample, with the help of the index.

Sequencing data analysis

Dada 2 package (Callahan et al., 2016) was used to generate import fasta files. Data were rarefied and filtered before analysis (Phyloseq package) (McMurdie and Holmes, 2016), removing sequences that were not present at least in 20% of all samples per treatment. The database silva_nr_v138 was used for taxonomic affiliation. Vegan (Oksanen et al.,2020) and Adegenet (Jombart, 2008) packages were used for non-metric multidimensional scaling analysis. Permanova analysis were performed to compare the 2 treatments and the ggplots2 package (Wickham, 2006) was used for plots.

Characterisation of SNCE

All solvents used in High-Performance Liquid Chromatography (**HPLC**) were of HPLC grade. Methanol and acetonitrile were purchased from Sigma-Aldrich. Deionized water from a Millipore Milli-Q water system was used to prepare mobile chromatographic phases.

To determine the proportion of polar/apolar compounds from SNCE, successive exhaustion dilutions have been performed with solvents ordered by increasing polarity (dichloromethane, methanol, and water). To do that, 500 mg of freeze-dried SNCE were weighed and solubilized in 10 mL of dichloromethane. After 5 minutes of sonication bath, the mixture was centrifuged for 10 minutes at 13 000 g and filtrated. The filtrate was recovered. Then 10 mL of dichloromethane was added to the solid deposit and the mixture was sonicated over 5 minutes, centrifuged for 10 minutes at 13000 g, and filtrated. The filtrate was recovered a second time and the procedure described was repeated a third time with dichloromethane. The same procedure was also performed 3 times with methanol, from the solid deposition obtained after the third filtration with dichloromethane. To finish, the same procedure was also performed 3 times with water, from the solid deposition obtained after the third filtration with methanol. Then, all the recovered filtrate per solvent were pooled in an evaporation flask and frozen at -80°C before lyophilization. The product obtained after lyophilization was weighed and the proportion of apolar/polar compounds was determined by

dividing the weight of the obtained freeze-dried material by the starting mass. The entire procedure was realized in triplicate.

Gaz chromatography-Mass spectrometry identification.

To analyze apolar compounds, 50 mg of freeze-dried SNCE were weighted and solubilized in 1 mL of cyclohexane. After 5 minutes of sonication bath (S30H, Elmasonic, Germany), the mixture was centrifuged for 10 minutes at 13,000 g. Then, 0.5 mL of the supernatant were added to 0.4 mL of anhydrous chloroform and 0.1 mL of N, O-Bis(trimethylsilyl)trifluoroacetamide (**BSTFA**) for derivatization. The mixture was then heated to 70°C for 20 minutes and cooled to room temperature for 10 minutes before analysis.

A Gas Chromatography-Mass Spectrometry (GCMS-QP2010SE, Shimadzu, Marne-la-Vallée, France) analysis was carried out with a ZB-5 column (30mx0.25mmx0.25 µm, Phenomenex) to identify apolar compounds from SNCE. Helium was used as carrier gas. 9.6 ml/min rate and split ratio 5:1 was applied to 1 µL injected samples. The column oven was programmed 80°C and 16°C/min to 320°C. Spectral data were compared to the NIST MS search program database (NIST 11, NIST 11s, FFNSC 2). Only compounds with an identification score higher than 95% from the NIST database were considered.

High Performances Liquid Chromatography analysis.

Freeze-dried SNCE were weighted (50 mg) and solubilized in 1 mL of methanol. After 5 minutes of sonication bath (S30H, Elmasonic, Germany), the mixture was centrifuged for 10 minutes at 13,000 g and the supernatant has been recovered for HPLC-MS^n analysis and HPLC-UV-**ELSD** (Evaporative light scattering detector) analysis. Regarding polar compounds, HPLC-UV-ELSD (Prominence-i C, ELSD-Sedex LT 90, Shimadzu, Marne-la-Vallée, France) analysis were performed following the methodology employed by Ledesma-Escobar *et al,* 2015 (Ledesma-Escobar C. A. et al., 2015) and using an Inertsil ODS-2 C18 analytical column (250 mm x 4.6 mm, 5µm). For ELSD conditions, the pressure of a carrier nitrogen gas and drift tube temperature were 3.5 bar and 40°C, respectively. Each analysis has been performed in triplicate on 5 different batches of SNCE. The injection volume was 10 µL. The mobile

phase was composed of 0.1% of formic acid in deionized water (A) and acetonitrile (B) at a flow rate of 1 mL/min. The initial condition was 4 % (B), 96 % (A). A gradient program was performed as follows: 4 % to 10 %B in 5 min, 10 % to 25 %B in 30 min; 25 % to 100 % B in 15 min, and constant 100 % B for 5 min before return to initial condition for 5 min. The objective here was to determine the HPLC profile of the citrus extract. The proportion of each polar compound family was also determined by relative quantification, from the ELSD profile data of the SNCE.

In parallel, HPLC-MS^n was performed to identify secondary polar metabolites from SNCE. Analyses were performed on a 2695 HPLC module coupled to a 2489 UV/visible detector (Waters, Saint-Quentin en Yveline, France) and an Esquire 3000 plus mass spectrometer (Bruker, Wissembourg, France), following the same HPLC conditions as described in HPLC-UV-ELSD part. Metabolites were identified using both positive and negative ionization modes. The ion source temperature was maintained at 340°C. A full scan was carried out within the m/z range 50-1500 using a collision energy of 1V. Spectral data were analyzed using DataAnalysis software (Bruker). Identification of the citrus extract compounds was carried out by comparing HPLC-MS^n analysis data of each compound detected with those reported in literature data, especially via the SciFinder database (American Chemical Society—SciFinder. Available online: http//sciFinder-n.cas.org/).

To complete, characterization of osidic compounds from SNCE were performed on Bioressources Imagerie, Biochimie & Structure (**BIBS**) instrumental platform (http://www.bibs.inra.fr/). Simple sugar content was determined using a **GC-FID** (Gaz Chromatography- Flame Ionization Detector) analyzer after acid hydrolysis of polymers in monomeric units. Simple sugars were then derivatized in alditols acetate before GC-analysis (Englyst and Cummings, 1988). In addition, carbohydrate acid content was determined by colorimetric dosage at 520 nm following the method developed by Blumenkrantz et Asboe-Hansen, 1973 (Blumenkrantz and Asboe-Hansen, 1973). Briefly, SNCE polymers were hydrolyzed into monomers using sulfuric acid (2N). Furfuric derivatives obtained from hydrolysis were coupled with meta-hydroxydiphenyl (**MHDP**) and then dosed with the spectrophotometer at 520 nm.

Statistical Analysis

Statistical analyses of growth performances data were performed by GraphPad Prism 7 software (GraphPad software, San Diego, CA, USA). The pen was considered as a statistical unit for all growth performances parameters except the weight in which the animal was used as a statistical unit. Statistical analyses were performed by Analysis of variance (ANOVA) or Kruskal-Wallis test when data were non parametric. Shapiro-Wilk normality test was performed to determine if data were parametric or not. Tukey post hoc were performed for non-parametric for multiple comparisons. Statistical significance was considered at $P < 0.05$. Regarding microbiota analysis, statistical analyses were performed using R software (R Development Core Team (2008)).

RESULTS

Standardized natural citrus extract Characterisation

Results from successive exhaustion dilution done in triplicate in 5 different batches showed that SNCE contained on average 0.38 % of apolar content (Table 3). GC-MS analysis confirmed the presence of stearic and palmitic acids in the SNCE.

Concerning polar compounds, LC-MS/MS analysis allowed to identify 35 secondary metabolites among polyphenols (flavones, flavanones, and flavanols), carboxylic acids, hydroxycinnamic acids, phenolic acids, and coumarin (Figure 1, Table 4).

Regarding polar compounds, the osidic part represents 51.48 % of the SNCE on average (Table 3). GC-FID analysis performed in duplicate allows to identify 6 simple sugars among rhamnose, arabinose, xylose, mannose, glucose, and galacturonic acids (Table 5).

Growth performances

The effects of different dietary experimental diets on growth performance are presented in Table 6. SNCE supplementation affects the bodyweight of broiler chickens at d 42 ($P = 0.004$, Anova). Broiler chickens that received feed supplemented with 250 ppm of SNCE were heavier compared to the control one ($P = 0.026$, Tukey). Similarly, broiler chickens supplemented with 2,500 ppm of SNCE

were also heavier than the control one (P = 0.001, Tukey). No statistical difference was observed between broiler chickens from SNCE 250 and SNCE 2,500 treatments in terms of body weight. Moreover, the feed intake of broiler chickens fed with 2,500 ppm of citrus extract was higher than the feed intake from the CTL treatment (P = 0.037, Tukey). SNCE supplementation did not affect other growth performances such as FCR (P = 0.661, Anova), mortality (P = 0.258, Anova) and litter score (P = 0.919, Anova).

Microbiota analysis

As expected, no difference was observed regardless of the level of supplementation on the cecal microbiota composition of broiler chickens at d 7 (P = 0.191) (Figure 2a). In fact, the non-metric multidimensional scaling (**NMDS**) performed didn't allow to separate samples from the 3 treatments. At d 42 SNCE supplementation didn't affect the microbiota composition of broiler chickens (P = 0.726). In fact, the non-metric Multidimensional Scaling (**MDS**) using Bray-Curtis matrix distance also didn't allow to separate supplemented samples from supplemented broiler chickens (250 and 2,500 ppm) from samples coming from unsupplemented broiler chickens (Figure 2b). However, the MDS analysis using Bray-Curtis distances revealed differences between the cecal microbiota of broiler chickens from d 7 and d 42 (P < 0.01) (Figure 2c) as would be expected. Major differences belong to the *Ruminococcaceae* whose proportion increase between d 7 and d 42, contrary to the *Lachnospiraceae* whose proportion decreases during this same period. *Lactobacillaceae* proportion was also more abundant at d 42 compared to d 7 **(**Figure 3a and Figure 3b). Regarding the composition at d 42, *Lactobacillus*, *Blautia,* and *Faecalibacterium* were the three most abundant genus in the ceca sample (Figure 3b), possibly reflecting well-balanced microbiota and good intestinal health from broiler chickens. Among all samples, the highest average number of *Lactobacillus* was observed in the experimental treatments fed with SNCE.

DISCUSSION

Characterization of SNCE

In the present study, characterization performed using 5 different batches of SNCE allowed to identify more than 99 % of the composition of the product. According to the results presented in Table 3, there is no inter-batches variation, confirming the standardization of the SNCE. This may partially explain the constant results observed on broiler chickens' growth performances regardless of the region and experimental period (Djezzar et al., 2017; Juin et al., 2003).

The osidic fraction of the citrus extract represents a major part of the product, with more than 98 % of citrus extract dry matter. The characterization of the structure was not complete due to the complex chemical composition of pectin, from which they come. These results are in contrast with the finding of Rafiq *et al*, who estimated the proportion of dietary fiber (which includes pectin derivative product) on different citrus extracts (Rafiq et al., 2016) between 44.2 % and 70.4 % of dry matter. SNCE process explains its richness in POS. Indeed, during manufacturing process of SNCE, the extract is standardized to achieve this level of fiber. Thus, explaining the high proportion of POS in the SNCE compared to the findings of Rafiq and colleagues.

Citrus extracts used in animal nutrition are mostly byproducts from industries such as juice canning industries (Alsaied et al., 2017) and essential oils industries (Chavan et al., 2018). Usually, they do not undergo any processing neither standardization, which may explain the described variability of their composition in scientific literature. Citrus byproducts have numerous applications such as ingredients for animal feed, the production of bio-fuel, pectin production, or the production of biodegradable packages (Chavan et al., 2018). During the process of SNCE manufacturing, a step of essential oil extraction is made. This explains the absence of these compounds in the SNCE, contrary to the literature reports (Ben Hsouna et al., 2017; Bendaha et al., 2016).

Characterization performed on SNCE may help to explain the mechanism of action behind the observed effect of SNCE on broiler chickens' growth performances. In fact, some molecules identified in SNCE have already been studied for their potential activity on health and performance. For

example, it's important to notice that pectic oligosaccharides present in SNCE are well described for their prebiotic effect (Gullón et al., 2013; Míguez et al., 2016). Their effects on the prevention against pathogenic bacteria and toxins have also been described (Michel Combo et al., 2011). These two pectic oligosaccharides effect are usually correlated with better performances in broiler chickens (Ma et al, 2019).

Regarding SNCE secondary metabolites, polyphenols which represent the major part of them, are also described for their beneficial effect on different compartments of the gut and intestinal microbiota. For instance, Unno and colleagues (Unno et al., 2015) demonstrated that the citroflavonoids of the citrus extracts promote the production of short-chain fatty acids in the colon, which results in pH diminution and pathogenic bacteria inhibition. Among the polyphenols identified from SNCE, eriocitrin, naringin, and hesperidin, which were among the most abundant, are particularly interesting. In fact, Gwiazdowska *et al* found that hesperidin and naringin stimulated the growth of *Bifidobacterium bifidum* at a concentration of 2 µg/mL (Gwiazdowska et al., 2015). Similarly, Estruel-Amades *et al* showed that hesperidin supplementation at 100 or 200 mg/kg three times per week results in an increase of *Lactobacillus* proportion in intestinal microbiota (Estruel-Amades et al., 2019). Hesperidin has also shown antioxidant properties and effect on broiler chicken fatty acids profile, reducing saturated fatty acids proportion in chicken meat and improving polyunsaturated fatty acids and omega n-6 content (Hager-Theodorides et al., 2021). These modifications of the fatty acids profiles were correlated with a reduction of the atherogenicity and thrombogenicity indices (Hager-Theodorides et al., 2021). In addition, Parkar and his team (Parkar et al., 2008) demonstrated the ability of naringenin, the aglycone of naringin, to inhibit the growth of some pathogenic strains including *E.coli*, *S.aureus*, and *S.typhimurium* in a concentration range of 62.5 to 125 µg/mL. The role of eriocitrin as antioxidant (Miyake et al., 2000) and antibacterial compound (Makni et al., 2018) has also been shown. Moreover, eriocitrin has recently shown positive effects on oxidative damage induced by Lipopolysaccharides from pathogens (Carvalho et al., 2021). As hesperidin, naringin, and eriocitrin, some other compounds identified in SNCE, such as

rutin, have already shown prebiotic effects, according to literature (Duda-Chodak, 2012; Stevens et al., 2019). As growth performances were enhanced in this study by SNCE supplementation and no effect was observed on cecal microbiota, we suggest that there was a possible effect on other parts of the intestine.

The higher body weight observed on broiler chickens fed with SNCE can be explained by other indirect effects of SNCE active compounds on the gut structure and/or motility. In fact, intestinal motility plays an important role in the assimilation of nutrients (Fändriks, 2017). A major compound of SNCE, hesperidin, is well documented as having a myorelaxant effect (Mendel et al., 2016). In fact, Mendel et al showed that hesperitin, the aglycone of hesperidin, induce myorelaxation on the jejunum of rats, which led to intestinal transit slowdown. By slowing down the intestinal transit, the contact time between the feed and the intestines is longer which may result in better absorption of nutrients and feed valorization. Further studies on chicken's intestines should be performed to confirm this hypothesis. In addition, Djezzar and colleagues have already shown an increase of the intestinal length and villi on broiler chickens, reflecting a larger absorption surface and a better feed valorization as consequences of SNCE supplementation (Djezzar et al., 2017).

Effect of SNCE on growth performances of broilers chickens

This study aimed to confirm the effect of SNCE on growth performances of broiler chickens and to understand its mode of action, regarding its composition and the possible effect on microbiota. The results show that supplementing a broiler chicken's diet with SNCE increased their bodyweight at d 42 ($P < 0.01$), compared to the ones fed with a standard diet without supplementation. The higher the concentration was, the greater the final bodyweight is. However, no dose effects of SNCE were observed in this trial. Boumezrag and colleagues also demonstrate a beneficial effect of this citrus extract on broiler chickens performances, showing an effect of SNCE supplementation at 250 ppm on live weight and carcass yield (Boumezrag et al., 2018). Our results are also in accordance with Juin *et al*, who also indicate an effect of SNCE on bodyweight and FCR of broiler chickens (Juin et al., 2003). A meta-analysis published recently confirmed these results (Cisse

et al., 2019). Taken together, these trials confirmed that SNCE can be a viable long-term alternative in substitution to AGP to support animal growth and feed efficiency. On the other hand, many results from published studies did not show positive effects of citrus/citrus extract supplementation on broiler chickens performances (Abbasi et al., 2015; Akbarian et al., 2013; Alzawqari et al., 2016; Nazok et al., 2010; Seidavi et al., 2018). Mourão and colleagues show that there are even negative effects on bodyweight average daily gain, feed conversion ratio, and carcass weight and yield of broiler chickens due to dietary citrus supplementation in feed (Mourão et al., 2008). This variability is not surprising. In fact, characterization of the citrus/citrus extract products data are often missing. As stated by Moset *et al*, the variability of the citrus composition may be strongly linked to the variability of the observed effect on broiler chickens (Cisse et al., 2020; Moset et al., 2015). The SNCE evaluated here is standardized by its manufacturing process but also in terms of concentration of some active compounds such as total phenolic compounds and some citroflavonoids (Hesperidin, Eriocitrin). The standardization of the SNCE was demonstrated by HPLC analysis carried out on five batches in this study. This fact may be at the origin of the reproducible results obtained with the SNCE by different authors and in different experimental conditions.

Effect of SNCE on microbiota

The obtained results showed that the microbiota composition of broiler chickens evolved between d 7 and d 42. The age of the broiler chickens has already been described as a variation factor for microbiota composition (Gabriel et al., 2005). As described by Lu *et al*, the proportion of *Lactobacillaceae* and *Ruminococcaceae* increased from d 7 to d 49 (Lu et al., 2003). Ranjiktar *et al* also observed a decrease of *Lachnospiraceae* proportion and an increase of *Ruminococcaceae* proportion on broiler chickens' ceca, from d 8 to d 36 (Ranjitkar et al., 2016). The different diets between production stages may probably explain these variations in cecal microbiota composition (Apajalahti et al., 2001). Other parameters such as the environment during the first days (Gabriel 2016), the immune system (Neish, 2009) and the nervous system (Collins et al., 2012) may also explain these results.

Moreover, the fine characterization of the SNCE showed the presence of many compounds such as pectic oligosaccharides, hesperidin, and naringin, previously described for their modulation effect on microbiota. For example, Gómez *et al* showed that Pectic oligosaccharides (POS) from citrus can be responsible for the promotion of Bifidobacteria and lactic bacteria growth (Gómez et al., 2014). Surprisingly, we did not observe any effect of SNCE supplementation in caecal microbiota. Our results are also in contradiction with the finding of Yu and colleagues who demonstrate that dietary supplementation with citrus extract improves the intestinal barrier function by modulating the microbial composition of broiler (Yu et al., 2019). These observed effects were positively correlated with growth performances improvement of broiler. The type of citrus extract, the feed composition, and also the inclusion rate (250 ppm Vs 10 ppm) can explain partially the difference observed by the cited authors compared to our results. Sampling methods and area are others possible explanations for these differences. Indeed, whereas the cecal microbiota was analyzed in our study, Yu et al analyzed a blend of cecal and ileal content (Yu et al., 2019). Similarly, Ebrahimi *et al* have also shown a correlated effect of *Citrus sinensis* supplementation between broiler chickens microbiota and growth performances (Ebrahimi et al., 2015). This highlights the fact that microbiota data are complex at harvest, treatment, and analyses. That's why special consideration must be given to their interpretation and comparison.

Dietary supplementation of SNCE to another species, namely pigs, during a shorter period revealed an important impact on fecal microbiota composition (Cisse et al., 2020). It should be noticed that in this cited study, the supplementation period, the inclusion rate, and the sampling area were different from the actual broiler chicken study. Differences may also be explained by the intestinal transit time which is different between sows and broilers.

CONCLUSION

Standardized natural citrus extract supplementation improved growth performances of broiler chickens. These effects, in this study, are not correlated with the cecal microbiota modulation. The fine characterization of the SNCE has shown that it has a stable composition from batch to batch. Moreover, SNCE composition allows to have new targeted hypotheses to better understand the observed effects. Based on the obtained results, we suggest the use of SNCE as a reliable natural feed additive to enhance broiler chicken performances.

DISCLOSURES

The authors with † work within the research and development department of Nor-Feed SAS. Nor-Feed SAS commercializes a product called Nor-Spice AB which is the Standardized natural citrus extract.

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Figure legend

Fig. 1. UV 280 nm (Blue) and ELSD profiles (Red) of the SNCE (50 mg/mL). Black circle represents the osidic fraction of the citrus extract.

Fig. 2. Comparison of cecal microbiota of broiler chickens at d 7 according to supplementation (a) – Comparison of cecal microbiota of broiler chickens at d 42 according to supplementation (b) Multidimensional scaling was performed using Bray-Curtis matrix distance. The *P*-value between the group was 0.191 (a) and 0.726 (b). – Comparison of cecal microbiota of broiler chickens of any group between d 7 and d 42 (c) ($P < 0.01$).

Fig. 3. Top 15 bacterial composition of caeca samples from each group at genus level at d 7 (a) and d 42 (b). Rarefaction were performed and data were filtered, removing sequences which were not present at least in 20% of all sample per group.

TABLES

Table 1 : Composition and calculated characteristics of the diets used during the experiment. d: digestible.

Item	Starter	Grower	Finisher
Ingredients (g/kg)			
Corn	358.05	231.15	184.30
Wheat	250.00	400.00	500.00
Soybean meal (48% CP)	267.00	214.00	155.00
Rapeseed meal (32,5% CP)	30.00	50.00	50.00
Soya hulls	33.00	30.00	30.00
Palm oil	0.00	20.00	30.00
Soybean oil	10.00	9.00	7.00
Vitamin & trace mineral premix	5.00	5.00	5.00
Limestone	15.00	12.70	11.50
Monocalcium phosphate	15.20	12.30	10.60
Salt	1.50	1.50	1.60
NaHCO3	3.50	3.50	3.00
L-Lys·HCl	3.85	3.65	4.00
DL-Met	3.30	2.90	2.75
L-Thr	1.85	1.65	1.70
L-Val	0.95	0.85	1.00
L-Ile	0.70	0.75	1.05
L-Arg	1.10	1.05	1.50
Total	1,00	1,00	1,00
Metabolizable energy (kcal/kg)	2,83	2,93	3,00
Crude protein %	20.71	19.43	17.51
dLys %	1.15	1.04	0.93
dMet/dLys	51	51	52
dMet+Cys/dLys	74	76	78
dArg/dLys	107	107	107
dThr/dLys	67	67	67
dTrp/dLys	17	18	17
dVal/dLys	75.00	76.00	76.00
dIle/dLys	67.00	68.00	69.00
Ca %	0.90	0.78	0.70
Available P %	0.45	0.39	0.35

Table 2 : Composition of the vitamin & trace mineral premix.

Nutrient	Premix supplies per kg of feed	Premix supplies per kg of premix
Vitamin A (retinyl acetate)	10,000 IU	2,000,000 IU
Vitamin D3 (cholecalciferol)	2,500 IU	500,000 IU
Vitamin E (dl-a-tocopherol)	50 mg	10,000 mg
Vitamin K3 (menadione)	1.5 mg	300 mg
Vitamin B1 (thiamin)	2.0 mg	400 mg
Vitamin B2 (riboflavin)	7.5 mg	1,500 mg
Vitamin B6 (pyridoxin-HCl)	3.5 mg	700 mg
Vitamin B12 (cyanocobalamin)	20 µg	4,000 µg
Niacin	35 mg	7,000 mg
D-pantothenic acid	12 mg	2,400 mg
Choline chloride	460 mg	92,000 mg
Folic acid	1.0 mg	200 mg
Biotin	0.2 mg	40 mg
Iron	80 mg	16,000 mg
Copper	12 mg	2,400 mg
Manganese	85 mg	17,000 mg
Zinc	60 mg	12,000 mg
Iodate	0.8 mg	160 mg
Selenium	0.15 mg	30 mg

Table 3: Standardized natural citrus extract composition.

	Batch 1 (%)	Batch 2 (%)	Batch 3 (%)	Batch 4 (%)	Batch 5 (%)	Mean (%)
Moisture content	47.20	47.20	47.20	47.80	47.52	47.38
Apolar compounds	0.42	0.42	0.42	0.26	0.35	0.38
Pectic oligo-saccharides	51.65	51.66	51.53	51.14	51.41	51.48
Polyphenols	0.71	0.67	0.77	0.72	0.61	0.70
Other secondary metabolites	0.02	0.06	0.09	0.08	0.11	0.07
Total	100.00	100.00	100.00	100.00	100.00	100.00

Table 4: Secondary metabolites identified from citrus extract tandem mass spectrometry analysis.

Rank	Rt (Min)	Compound	Neutral M	MS1	Mode	Chemical family	λ max (nm)
1	2.4	L-Asparagine	132	133	[M+H] +	Amino acid	
2	2.8	L-Proline betaine	143	144	[M+H] +	Amino acid	
3	5.0	Adenosine	267	268	[M+H] +	Nucleoside	260-274sh
4	5.3	L-Arginine	174	175	[M+H] +	Amino acid	
5	8.9	Vanillic acid	168	167	[M-H] -	Benzoic acid	296
6	11.0	N-Caffeoyltryptophan	366	365	[M-H] -	Propanoic acid	
7	12.9	3-Hydroxymethylglutaric acid	162	163	[M+H] +	Carboxylic acid	
8	15.9	Acid Hydroxycinnamoyl-O-glucoside	356	355	[M-H]-	Hydroxycinnamic acid	
9	17.6	Dihydroferulic acid-Glu	520	519	[M-H]-	Phenolic acid	277 311
10	19.1	Citric acid	191	190	[M-H]-	Carboxylic acid	
11	19.8	Vicenin 2	594	593 595	[M-H]- [M+H] +	Flavone	271 333
12	20.8	Stellarin 2	624	623 625	[M-H]- [M+H] +	Flavone	336 268sh
13	21.3	Eriodictyol -Glu-Rha-Glu	758	757	[M-H]-	Flavanone	276
14	23.6	3-(2-hydroxy-4-methoxyphenyl)-propanoic acid hexose	358	357	[M-H]-	Propanoic acid	
15	23.9	Dihydro-feruloyl-O-glucoside	358	357	[M-H]-	Phenolic acid	
16	27.0	Vitexin-O-xyloside	564	563 565	[M-H]- [M+H] +	Flavone	312
17	27.5	8'-hydroxyabscisic acid Glu	442	441	[M-H]-	abscisic acid	
18	28.3	Eriocitrin	596	595 597	[M-H]- [M+H] +	Flavanone	283
19	28.5-	Rutin	610	609	[M-H]-	Flavonol	
20	28.7	Kaempferol-3-O-Rutinoside	594	593	[M-H]-	Flavonol	256. 266.350
21	30.3	Scoparin / Diosmetin-Glu	462	461 463	[M-H]- [M+H] +	Flavone	345 263-267sh
22	31.2	Dihydroferulic acid-HMG-Glu	502	501	[M-H]-	Phenolic acid	
23	32.2	Limocitrin-Neo	654	653	[M-H]-	Flavonol	254 [1] / 261-275sh 308-327sh
24	32.9	Naringin	580	579 581	[M-H]- [M+H] +	Flavanone	262 322-328sh
25	33.1	Rhoifolin	579	577 579	[M-H]- [M+H] +	Flavone	259-278sh 314-327sh
26	33.2	Limocitrin-Glu-HMG-Glu	814	813	[M-H]-	Flavonol	251-261sh 310-314sh
27	33.7	Diosmin / Neodiosmin	608	607 609	[M-H]- [M+H] +	Flavone	268
28	33.9	Hesperidin	448	449	[M+H] +	Flavanone	285
29	35.6	Limocitrin-HMG-Glu	652	651 653	[M-H]- [M+H] +	Flavonol	252-271sh 352
30	38.7	Limocitrol-Glu-HMG	682	681 683	[M-H]- [M+H] +	Flavonol	263-273sh 339

31	40.4	P-coumaric acid	164	163	[M-H]-	Hydroxycinnamic acid	298.5 - 308.5
32	39.9	Limocitrol-Glu-HMG-Glu	796	795 797	[M-H]- [M+H] +	Flavonol	263-271sh 342
33	41.3	Oxypeucedanin hydrate	304	305	[M+H] +	Linear Furanocoumarins	335 271
34	41.5	Byakangelicol	316	317	[M+H] +	Linear Furanocoumarins	220
35	46.2	Vitexin	432	433	[M+H] +	Flavone	

Rank: pic number, Rt: retention time, MS1: mass spectrometry 1, [M-H]-: negative ionization mode, [M+H] +: positive ionization mode, sh: shoulder.

Table 5: POS composition of SNCE determined by Gas Chromatography- Flame Ionization Detector (GC-FID) analysis.

	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	AU	OT
Freeze-dried SNCE osidic composition (%dry matter)	0.30	ND	0.70	0.30	1.80	0.60	27.10	11.90	42.70

Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; AU: galacturonic acid; OT: total oses; ND: Not Determined.

Table 6: Standardized natural citrus extract dietary supplementation effect on zootechnical performances of broiler chickens at day 42.

Treatment	Control	SNCE 250	SNCE 2,500	Pooled SEM	*P*-value
Body weight (g)	3,288[a]	3,340[b]	3,364[b]	26.05	< 0.01
Daily feed consumption (g/chicken/day)	124..5[a]	124.9[a]	127.2 [b]	3.0	0.08
Feed conversion ratio	1.64 [a]	1.63 [a]	1.64 [a]	0.02	0.66
Mortality (%)	6.7 [a]	7.9 [a]	5.6 [a]	5.0	0.26
Litter score (Average)	2 [a]	2 [a]	2 [a]		0.92

Statistical analyses were performed by ANOVA or Kruskal Wallis test. when data were non-parametric. Statistical significance was considered at $P < 0.05$.

Figure 1

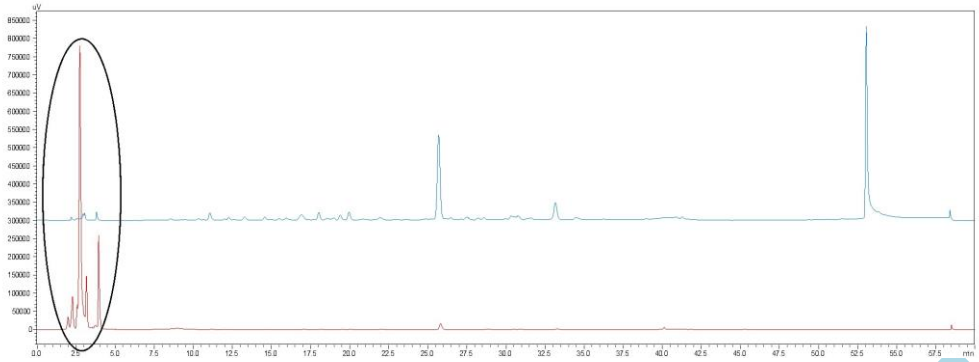

Figure 2

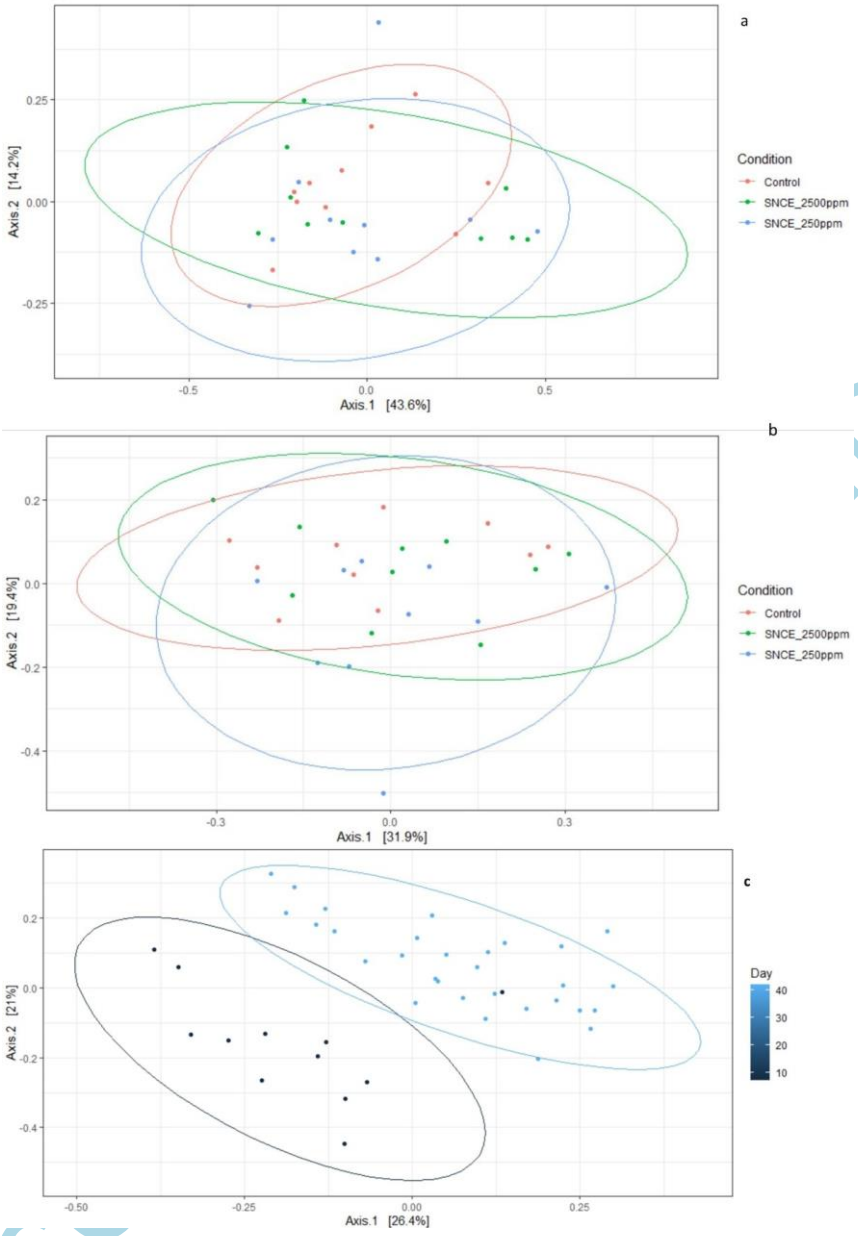

Figure 3