Effects of Stocking Density on Cecal Microbiota Diversity, Volatile Fatty Acids, and Serum Brain-Gut Peptides in Broiler Chickens: Postprint

Authors: Chang Shuangshuang, Liu Qingxiu, Zhang Minhong, Shi Yuxiang, Feng Jinghai

Date: 2018-12-24T00:00:00+00:00

Abstract

This study aimed to investigate the effects of stocking density on cecal microbiota diversity, volatile fatty acids, and serum brain-gut peptides in broiler chickens. A single-factor experimental design was employed, in which 144 healthy 22-day-old Arbor Acres broilers with similar body weight were selected and randomly allocated into 3 groups, with 6 replicates per group, and each replicate was housed in a single cage. Broilers from all groups were transferred to an environmental control chamber (ambient temperature: 21°C; relative humidity: 60%) for a 7-day acclimation period. The formal experiment commenced at 29 days of age; the floor area per cage was 0.64 m^2, and stocking densities were set at 6 birds per cage (half male and half female, Group I), 8 birds per cage (half male and half female, Group II), and 10 birds per cage (half male and half female, Group III). Ambient temperature (21°C) and relative humidity (60%) were maintained throughout the experimental period, which lasted for 14 days. The results demonstrated: 1) Group III exhibited the highest cecal microbiota richness and diversity, and this stocking density facilitated the colonization of Clostridium termitidis and Bacteroides vulgatus in the broiler cecum. 2) Stocking density had no significant effect on cecal volatile fatty acid concentrations ($P>0.05$). 3) On day 14 of the experiment, serum vasoactive intestinal peptide (VIP) concentration in Group III was significantly lower than that in Groups I and II ($P<0.05$), whereas serum 5-hydroxytryptamine and substance P concentrations did not differ significantly among the three groups ($P>0.05$). In summary, stocking density can alter cecal microbiota diversity and structure as well as serum vasoactive intestinal peptide concentration, but does not influence cecal volatile fatty acid concentrations.

Full Text

Effects of Stocking Density on Cecal Microflora Diversity, Volatile Fatty Acids, and Serum Brain-Gut Peptides in Broilers

CHANG Shuangshuang[1],[2], LIU Qingxiu[1], ZHANG Minhong[1]*, SHI Yuxiang[2], FENG Jinghai[1]

[1]State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China
[2]College of Agriculture, Hebei University of Engineering, Handan 056021, China

Abstract

This study investigated the effects of stocking density on cecal microflora diversity, volatile fatty acids, and serum brain-gut peptides in broilers. A single-factor design was adopted, and 144 healthy 22-day-old Arbor Acres broilers with similar body weight were randomly divided into three groups, each consisting of six replicates with single-cage housing. The broilers were transferred to an environmental control chamber at 21 °C and 60% relative humidity for a 7-day acclimation period. The formal experiment began when the broilers reached 29 days of age. The single cage area was 0.64 m^2, and stocking densities were set at 6 birds per cage (half male and half female, Group), 8 birds per cage (half male and half female, Group), and 10 birds per cage (half male and half female, Group). Environmental conditions of 21 °C and 60% relative humidity were maintained until the end of the 14-day trial. The results showed that: (1) Group exhibited the highest richness and diversity of cecal microflora, and this stocking density favored the colonization of *Clostridium termitidis* and *Bacteroides vulgatus* in the cecum. (2) Stocking density had no significant effect on cecal volatile fatty acid contents ($P > 0.05$). (3) On day 14 of the experiment, serum vasoactive intestinal peptide (VIP) content in Group was significantly lower than in Groups and ($P < 0.05$), while no significant differences were observed in serum 5-hydroxytryptamine or substance P contents among the three groups ($P > 0.05$). In conclusion, stocking density can alter cecal microflora diversity and structure as well as serum VIP content, but does not affect cecal volatile fatty acid concentrations in broilers.

Keywords: stocking density; cecal microflora; volatile fatty acid; brain-gut peptide; broiler

---

Introduction

The poultry industry has long prioritized stocking density to maximize output per unit area and economic benefits. While higher stocking densities can increase profitability, they negatively impact production performance, health, and

welfare. Previous studies have reported that high stocking density (24 birds/m²) significantly reduces body weight, average daily gain, and average daily feed intake in broilers. High density also affects gait scores and increases the incidence of leg dermatitis. Research has shown low similarity (33.1%–65.4%) in cecal microflora structure among different stocking densities (900, 675, 540, 450, and 380 cm²/bird), indicating substantial variation in cecal microbiota under different density conditions.

Volatile fatty acids, also known as short-chain fatty acids, are major metabolites and important signaling molecules produced by gut microflora. They not only stabilize microflora structure but also regulate intestinal immune motility and epithelial barrier function. Short-chain fatty acids can lower intestinal pH, promoting the growth of beneficial bacteria while inhibiting specific pathogenic colonization. Additionally, studies have demonstrated reciprocal interactions between intestinal microflora and brain-gut peptide secretion. For instance, *Bifidobacterium* quadruple viable tablets can significantly increase vasoactive intestinal peptide (VIP) levels while decreasing substance P (SP) levels. Brain-gut peptides such as 5-hydroxytryptamine (5-HT), VIP, and SP are known to be associated with gastrointestinal activity. However, research on how stocking density affects serum brain-gut peptides and cecal volatile fatty acids in broilers remains scarce. Therefore, this study aimed to investigate these effects to provide a theoretical basis for determining optimal stocking densities in broiler production.

Materials and Methods

1.1 Experimental Design

This experiment employed a single-factor design. One hundred forty-four healthy 22-day-old Arbor Acres broilers with similar body weight were randomly allocated to three groups, each comprising six replicates (one cage per replicate). All groups were transferred to an environmental control chamber maintained at 21 °C and 60% relative humidity for a 7-day acclimation period. The formal trial commenced when the broilers reached 29 days of age. The single cage area was 0.64 m², with stocking densities set at 6 birds per cage (half male and half female, Group), 8 birds per cage (half male and half female, Group), and 10 birds per cage (half male and half female, Group). Environmental conditions of 21 °C and 60% relative humidity were maintained throughout the 14-day experimental period. The trial was conducted in the environmental control chamber of the State Key Laboratory of Animal Nutrition, with automatic temperature and humidity control (accuracies of ±1 °C and ±7%, respectively), no airflow, and 24-hour lighting.

1.2 Experimental Diet

A corn-soybean meal basal diet was formulated according to NRC (1994) nutritional requirements. The composition and nutrient levels of the basal diet are presented in Table 1 .

1.3 Management

All broilers were raised in single-tier floor cages developed by our laboratory, with free access to feed and water and conventional immunization.

1.4 Sample Collection and Analysis

1.4.1 Blood Collection and Analysis On days 7 and 14 of the experiment, six broilers were randomly selected from each group (half male and half female, one bird per replicate) for venous blood collection. After standing for 2 hours, serum was collected by centrifugation at 3,000 rpm for 10 minutes and stored at -80 °C. Serum 5-HT, VIP, and SP contents were determined by enzyme-linked immunosorbent assay (ELISA).

1.4.2 Cecal Content Collection and Analysis 1.4.2.1 Sample Collection
On days 7 and 14, six broilers from each group (half male and half female, one bird per replicate) were euthanized by cervical dislocation. After whole-body disinfection, the abdominal cavity was opened, the intestine was isolated, and the ileocecal junction was ligated. The cecum was quickly transferred to a clean bench, opened with sterile scissors, and the contents were collected into sterile centrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80 °C.

1.4.2.2 Volatile Fatty Acid Measurement
Approximately 2 g of cecal digesta was weighed into a centrifuge tube, and 5 mL of ultrapure water was added. The mixture was vortexed for 3–5 minutes and centrifuged at 5,000 × g for 10 minutes. The supernatant (1 mL) was transferred to a plastic ampoule, mixed with 0.2 mL of 25% metaphosphoric acid, sealed, shaken, and placed in an ice bath for 30 minutes. After centrifugation at 10,000 × g for 10 minutes, the supernatant was collected for determination of acetic, propionic, butyric, and pentanoic acid contents.

1.4.2.3 DNA Extraction
Genomic DNA was extracted from samples using the Fast DNA™ SPIN Kit for Soil.

1.4.2.4 16S rDNA PCR Amplification
Using the extracted genomic DNA as template, bacterial universal primers GC-338F and 518R were used to amplify the 16S rDNA hypervariable region. Primer information is provided in Table 2 . The PCR reaction mixture (50 L) contained: 10× PCR buffer 5 L, dNTP Mixture (2.5 mmol/L) 3.2 L, Ex Taq (5 U/ L) 0.4 L, GC-338F (20 mol/L) 1 L, 518R (20 mol/L) 1 L, template DNA 50 ng,

and ddH O to 50 L. The PCR program was: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 55 °C for 45 s, 72 °C for 1 min; and final extension at 72 °C for 10 min. PCR products were purified using the OMEGA DNA Gel Extraction Kit. A Biometra T-gradient thermocycler and Bio-Rad Gel-Doc2000 imaging system were used.

1.4.2.5 DGGE Analysis

Ten microliters of PCR product were analyzed by DGGE using 7% polyacrylamide gel with a 35%–55% denaturing gradient at 150 V and 60 °C in 1× TAE buffer for 5 hours. After electrophoresis, gels were silver-stained: (1) fixed in fixative solution (50 mL ethanol, 2.5 mL glacial acetic acid, diluted to 500 mL) for 15 min; (2) washed with Milli-Q water for 20 s and 2 min; (3) stained in silver nitrate solution (1 g AgNO , 0.75 mL 37% formaldehyde, diluted to 500 mL) for 15 min; (4) washed with Milli-Q water for 20 s and 2 min; (5) developed in developer solution (7.5 g NaOH, 2.5 mL 37% formaldehyde, diluted to 500 mL) for 5–7 min; and (6) terminated in stop solution (50 mL ethanol, 2.5 mL glacial acetic acid, diluted to 500 mL).

1.4.2.6 Sequencing of Dominant DGGE Bands

Dominant DGGE bands were excised and re-amplified using primers 338F/518R. PCR products were purified, ligated into pMD18-T vector, transformed into DH5 competent cells, and positive clones were sequenced. Sequences were compared with GenBank database to identify bacterial types, with three clones sequenced per band.

1.5 Statistical Analysis

Data were analyzed using SAS 9.2 software for one-way ANOVA, with Duncan' s multiple comparison test. Differences were considered significant at $P < 0.05$. DGGE profile diversity was analyzed using Quantity One software.

Results

2.1.1 PCR-DGGE Fingerprint Analysis of Cecal Microflora

As shown in Figure 1 [Figure 1: see original paper] and Figure 2 [Figure 2: see original paper], on day 7, Groups and had more cecal microflora bands than Group , while on day 14, Group had more bands than Groups and . Table 3 shows that similarity indices among different stocking densities ranged from 54.0% to 80.3% within the same experimental period. On day 7, the lowest similarity (54.0%) occurred between Groups and , whereas on day 14, the highest similarity (80.3%) was between Groups and . Overall, the effect of stocking density on cecal microflora structure changed with broiler age, with lower similarity between Groups and than between Groups and , indicating greater differences in cecal microflora structure between Groups and .

2.1.2 Diversity Analysis of Cecal Microflora Structure

Table 4 shows that cecal microflora diversity indices varied with stocking density. On day 7, Shannon and Simpson indices were 2.441 and 0.910 for Group , 1.921 and 0.850 for Group , and 2.453 and 0.912 for Group , respectively. On day 14, these indices were 2.649 and 0.926 for Group , 2.659 and 0.927 for Group , and 2.787 and 0.936 for Group . Throughout the experiment, Group exhibited the highest Shannon and Simpson diversity indices, suggesting that a stocking density of 10 birds per 0.64 m^2 provided optimal cecal microflora diversity and richness.

2.1.3 Analysis of Specific and Common Microflora

Two specific bands and four common bands were excised from the 16S rDNA V3 region PCR-DGGE fingerprints. As shown in Figure 1 and Table 5 , common bands were detected in all three groups on both days 7 and 14: band 3 (*Holdemanella biformis*), band 4 (*Bacteroides uniformis*), band 5 (*Eisenbergiella massiliensis*), and band 6 (*Ruminococcus faecis*). Bands 1 (*Clostridium termitidis*) and 2 (*Bacteroides vulgatus*) were detected only in Group . All six sequences belonged to phyla Firmicutes or Bacteroidetes, with >90% similarity to GenBank database sequences.

2.2 Cecal Volatile Fatty Acid Contents

Table 6 shows that stocking density had no significant effect on cecal volatile fatty acid contents on days 7 or 14 ($P > 0.05$). However, Group tended to have slightly lower volatile fatty acid levels than the other two groups.

2.3 Serum Brain-Gut Peptide Contents

Table 7 shows that on day 7, stocking density had no significant effect on serum 5-HT, VIP, or SP contents ($P > 0.05$). On day 14, serum VIP content in Group was significantly lower than in Groups and ($P < 0.05$), while no significant differences in serum 5-HT or SP contents were observed among the three groups ($P > 0.05$).

Discussion

3.1 Effects of Stocking Density on Cecal Microflora Diversity

Intestinal microorganisms play crucial physiological roles in nutrient digestion and absorption, immune system development, and host energy metabolism. A balanced intestinal microflora structure can effectively inhibit pathogen invasion and improve animal health and performance. However, under stress conditions (including excessively high or low stocking density), this balance can be disrupted, potentially leading to pathogen proliferation and disease. Guardia et

al. reported that stocking density significantly affected digestive tract microflora in broilers at 3 weeks of age, with the most pronounced changes in crop and cecal microflora (similarity analysis R-values of 0.77 and 0.69, respectively, P 0.05). At 6 weeks, significant differences persisted (R-values of 0.52 and 0.27, respectively, P 0.05). Our results showed that Group had higher cecal microflora richness and similarity than Groups and , indicating that a stocking density of 10 birds per 0.64 m^2 favored cecal microflora growth and balance.

The predominant bacterial phyla in chicken cecum are Firmicutes, Bacteroidetes, and Proteobacteria. Studies have reported that increasing stocking density above 450 cm^2/bird causes beneficial bacteria (*Lactobacillus gastricus* and *Lactobacillus alvi*) to disappear from the duodenum of caged layers, adversely affecting intestinal microflora balance. Our study found that *Clostridium termitidis* and *Bacteroides vulgatus* were specifically detected in Group but not in the other groups. *Clostridium termitidis* belongs to Firmicutes, the dominant phylum in the hindgut microbiota comprising 60%–70% of the community, with butyrate-producing bacteria playing important roles in intestinal epithelial cell development. *Bacteroides vulgatus* belongs to Bacteroidetes, the largest group of Gram-negative bacteria in the intestine that produces enzymes degrading plant cell walls, thus participating in digestive functions.

3.2 Effects of Stocking Density on Cecal Volatile Fatty Acid Contents

Short-chain fatty acids such as acetic, propionic, and butyric acids are major products of carbohydrate and protein fermentation in the intestine, with over 95% being absorbed and metabolized by the host. These compounds perform important physiological functions including regulation of intestinal microflora, maintenance of fluid and electrolyte balance, energy provision, and nutrition for intestinal epithelial cells. In poultry, volatile fatty acids are present throughout the digestive tract, with the highest concentrations in the cecum. Our results showed no significant effect of stocking density on cecal volatile fatty acid contents, though Group tended to have lower levels, possibly due to reduced populations of volatile fatty acid-producing microorganisms in the cecum.

3.3 Effects of Stocking Density on Serum Brain-Gut Peptide Contents

Brain-gut peptides are neuropeptide hormones distributed in both the gastrointestinal tract and central nervous system. They act as neurotransmitters or modulators on receptors of gastrointestinal sensory nerve endings or smooth muscle cells, regulating gastrointestinal sensation and motility. Circulating brain-gut peptide hormones can enter the hypothalamus via blood circulation and directly act on related chemoceptors in the arcuate nucleus to produce biological effects.

5-HT is primarily produced by intestinal endocrine cells and participates in regulating intestinal motility and sensation. Early-life microbiota deficiency reportedly increases plasma tryptophan levels, while *Bifidobacterium* can affect tryptophan metabolism. Oral administration of *Bifidobacterium infantis* can

induce increased plasma concentrations of dopamine and 5-HT in rats. VIP reduces smooth muscle tone, inhibits gastrointestinal motility, and stimulates secretion. It also improves intestinal mucosal microcirculation and environment, provides nutrients and oxygen to epithelial cells, removes harmful substances such as excess oxygen free radicals, reduces lipopolysaccharide-induced mucosal damage, and promotes beneficial bacterial colonization to restore normal microflora balance. Substance P is a tachykinin that increases gastrointestinal motility, strongly promotes smooth muscle contraction, enhances colonic mass movement, stimulates water and electrolyte secretion from small intestinal and colonic mucosa, dilates gastrointestinal blood vessels, increases vascular permeability, causes plasma extravasation, and participates in inflammatory and immune responses.

Our study found that serum VIP content in Group was significantly lower than in Groups and only on day 14, which may be related to changes in the intestinal environment and microflora structure requiring further investigation. Under thermoneutral conditions (21 °C), stocking density had no significant effect on serum 5-HT or SP contents.

Conclusion

1. Group exhibited higher cecal microflora richness and similarity than Groups and , and this stocking density favored the colonization of *Clostridium termitidis* and *Bacteroides vulgatus*.
2. Stocking density had no significant effect on cecal volatile fatty acid contents in broilers.
3. Serum VIP content in Group was significantly lower than in Groups and , but stocking density had no significant effect on serum 5-HT or SP contents.

References

[1] ZHANG F S. Integrated management strategies and techniques for nutrient resources in China[C]//Circular Agriculture and New Rural Construction —Proceedings of 2006 Annual Academic Conference of Chinese Association of Agricultural Science Societies. Beijing: Chinese Association of Agricultural Science Societies, 2006: 371-374.

[2] SHAO D, ZHANG S, SHI S R, et al. Effects of stocking density on production performance, immune organ indices, and meat quality of yellow-feathered broilers[J]. Chinese Journal of Animal Nutrition, 2015, 27(4): 1230-1235.

[3] TONG H B, LU J, ZOU J M, et al. Effects of stocking density on growth performance, carcass yield, and immune status of a local chicken breed[J]. Poultry Science, 2012, 91(3): 667–673.

[4] ESTEVEZ I. Density allowances for broilers: where to set the limits?[J]. Poultry Science, 2007, 86(6): 1265–1272.

[5] DOZIER W A, THAXTON J P, BRANTON S L, et al. Stocking density effects on growth performance and processing yields of heavy broilers[J]. Poultry Science, 2005, 84(8): 1332–1338.

[6] ARNOULD C, FAURE J M. Use of pen space and activity of broiler chickens reared at two different densities[J]. Applied Animal Behaviour Science, 2003, 84(4): 281–296.

[7] ZHAO Q Y. Effects of stocking density on small intestinal histology and cecal bacterial populations in caged layers[D]. Master' s thesis. Daqing: Heilongjiang Bayi Agricultural University, 2015.

[8] XU W L, LU G, LIANG S J, et al. Short-chain fatty acid-mediated microbiota-host interaction and irritable bowel syndrome[J]. World Chinese Journal of Digestology, 2015, 36(23): 5815–5822.

[9] ESWARAN S, MUIR J, CHEY W D. Fiber and functional gastrointestinal disorders[J]. The American Journal of Gastroenterology, 2013, 108(5): 718–727.

[10] SILK D B A, DAVIS A, VULEVIC J, et al. Clinical trial: the effects of trans-galactooligosaccharide prebiotic on faecal microbiota and symptoms in irritable bowel syndrome[J]. Alimentary Pharmacology & Therapeutics, 2009, 29(5): 508–518.

[11] FUKUDA S, TOH H, HASE K, et al. Bifidobacteria can protect from enteropathogenic infection through production of acetate[J]. Nature, 2011, 469(7331): 543–547.

[12] BAI Y, HU Y X, CHEN J W, et al. Establishment of the bacteria-brain-gut axis theoretical system[J]. Journal of Southeast University (Medical Science Edition), 2016, 35(5): 781–785.

[13] FU L X. Therapeutic efficacy of Bifidobacterium quadruple viable tablets in patients with diarrhea-predominant irritable bowel syndrome and its effect on serum brain-gut peptide levels[J]. Chinese Journal of Microecology, 2017, 29(2): 179–181.

[14] CHEN Q. Clinical study on the effect of umbilical moxibustion with herbal medicine on serum brain-gut peptides in irritable bowel syndrome patients with spleen-qi deficiency[D]. Master' s thesis. Jinan: Shandong University of Traditional Chinese Medicine, 2011.

[15] ZHANG M H, SU H G, FENG J H. Method and special device for collecting data to establish a broiler living environment comfort evaluation model: China, CN103404447A[P]. 2015–11–18.

[16] PAN D, YU Z T. Intestinal microbiome of poultry and its interaction with host and diet[J]. Gut Microbes, 2014, 5(1): 108–119.

[17] XIAO Y P, YANG C M, DAI B, et al. Effects of *Clostridium butyricum* on cecal microflora structure in broilers based on high-throughput sequencing[J]. Acta Agriculturae Zhejiangensis, 2017, 29(3): 373–379.

[18] SEKELJA M, RUD I, KNUTSEN S H, et al. Abrupt temporal fluctuations in the chicken fecal microbiota explained by its gastrointestinal origin[J]. Applied and Environmental Microbiology, 2012, 78(8): 2941–2948.

[19] GUARDIA S, KONSAK B, COMBES S, et al. Effects of stocking density on the growth performance and digestive microbiota of broiler chickens[J]. Poultry Science, 2011, 90(9): 1878–1889.

[20] QU A, BRULC J M, WILSON M K, et al. Comparative metagenomics reveals host-specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome[J]. PLoS One, 2008, 3(8): e2945.

[21] YU J M, WANG Q J, LIU B L, et al. Effects of different stocking densities on duodenal microflora in caged layers[J]. Chinese Journal of Animal Nutrition, 2016, 28(3): 899–907.

[22] PRYDE S E, DUNCAN S H, HOLD G L, et al. The microbiology of butyrate formation in the human colon[J]. FEMS Microbiology Letters, 2002, 217(2): 133–139.

[23] PENG Q Q, WANG X M, ZHANG M H, et al. Effects of continuous moderate heat stress on cecal microflora diversity in broilers[J]. Scientia Agricultura Sinica, 2016, 49(1): 186–194.

[24] GIBSON G R, MACFARLANE G T. Human colonic bacteria: role in nutrition, physiology and pathology[M]. Boca Raton: CRC Press, 1995.

[25] CHEN Y, CAO Y S, LIU X H. Short-chain fatty acids and intestinal microflora[J]. Jiangxi Science, 2006, 24(1): 38–40, 69.

[26] ANNISON E F, HILL K J, KENWORTHY R. Volatile fatty acids in the digestive tract of the fowl[J]. British Journal of Nutrition, 1968, 22(2): 207–216.

[27] DOCKRAY J. Cholecystokinin and gut–brain signalling[J]. Regulatory Peptides, 2009, 155(1/2/3): 6–10.

[28] TOMITA R. Regulation of vasoactive intestinal peptide and substance P in the human pyloric sphincter[J]. Hepato-Gastroenterology, 2009, 56(94/95): 1403–1406.

[29] DESBONNET L, GARRETT L, CLARKE G, et al. The probiotic *Bifidobacteria infantis*: an assessment of potential antidepressant properties in the rat[J]. Journal of Psychiatric Research, 2008, 43(2): 164–174.

[30] DESBONNET L, GARRETT L, CLARKE G, et al. Effects of the probiotic *Bifidobacterium infantis* in the maternal separation model of depression[J].

Neuroscience, 2010, 170(4): 1179–1188.

[31] HAMRMAR A J, FAHRENKRUG J, GOZES I, et al. Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR Review[J]. British Journal of Pharmacology, 2012, 166(1): 4–17.

[32] WU M Y, HU T M. Research progress on vasoactive intestinal peptide and digestive diseases[J]. World Chinese Journal of Digestology, 2012, 20(16): 1453–1457.

[33] CAMPOS-SALINAS J, CAVAZZUTI A, O' VALLE F, et al. Therapeutic efficacy of stable analogues of vasoactive intestinal peptide against pathogens[J]. Journal of Biological Chemistry, 2014, 289(21): 14583–14599.

[34] CHEN X M, ZHANG Y H, WU Y L. Changes of substance P and vasoactive intestinal peptide in colonic mucosa of irritable bowel syndrome patients[J]. Journal of Clinical Medicine in Practice, 2008, 12(11): 31–33.

Note: Figure translations are in progress. See original paper for figures.